SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF SWITCHGRASS BY THERMOTOLERANT *KLUYVEROMYCES MARXIANUS* IMB3: EFFECT OF ENZYME LOADING, TEMPERATURE AND OPERATING MODE

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CHAPTER I

INTRODUCTION

Depletion of fossil fuels and rapid growth of automobile usage clearly shows the need for alternative fuels. According to the Energy Information Administration (EIA), the United States imports approximately 19 million barrels of petroleum per day and is the leading consumer of oil in the world (Anonymous, 2010). Use of alternative sources of energy has been a hot topic around the world. Significant research has been done in the field of ethanol and its usage as automobile fuel (Hahn-Hägerdal et al., 2006; Hansen et al., 2005; Yüksel & Yüksel, 2004). Ethanol could be produced from a variety of resources, such as food grains and lignocellulosic biomass. The Energy Independence and Security Act of 2007 requires the production of 36 billion gallons of ethanol by the year 2022 among which 21 billion gallons must come from non-corn-based ethanol (Anonymous, 2009b). Several concerns, such as increased cost of food products, have been raised because of the use of corn and other food resources for fuel production (Naylor et al., 2007; Pimentel et al., 2009). Thus, the use of cellulosic feedstocks derived from non-food resources, such as switchgrass, for ethanol production would be beneficial. Moreover, reduction in greenhouse gas (GHG) emissions was observed when ethanol derived from lignocellulosic material was used as a fuel (Farrell et al., 2006) . Lignocellulosic biomass consists primarily of cellulose, hemicelluloses and lignin.

However, the composition of each component varies with the feedstock used (Bals et al., 2010; Huang et al., 2009; Mosier et al., 2005). Cellulose is a polymer of glucose molecules linked by β-1,4-glycosidic bonds and is protected by lignin, which is a polymer of phenylpropanoid units (Mosier et al., 2005). Hemicellulose is a heteropolymer of D-glucose, D-xylose, D-galactose, D-mannose and D-arabinose units linked via β-1,4-glycosidic bonds (Cheng, 2009)

The biological process of ethanol production from lignocellulosic material requires three steps: (1) pretreatment of lignocellulosic material, (2) saccharification of polysaccharides using enzymes to fermentable sugars and (3) fermentation of sugars to ethanol. A typical lignocellulosic ethanol production process is shown in Fig. 1.1. In order to achieve high conversion efficiency of cellulosic biomass into ethanol, pretreatment of cellulosic biomass is needed prior to the fermentation process. A typical pretreatment process should decrease the crystallinity of cellulose, remove hemicelluloses and increase the surface area of biomass (Mosier et al., 2005), which would eventually reduce the use of enzymes to hydrolyze cellulose (Mosier et al., 2005; Wyman, 1999).

Ethanol can be obtained using different schemes, such as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and simultaneous saccharification and co-fermentation (SSCF). However, the products formed in SHF, such as cellobiose and glucose, can inhibit cellulase enzyme and fermenting microorganisms (Alfani et al., 2000). With SSF, the glucose produced during hydrolysis is metabolized directly by the microorganism, thereby reducing product inhibition (Alfani et al., 2000; Stenberg et al., 2000; Wingren et al., 2003; Xiao et al., 2004). Moreover, SSF has several other advantages, such as reduced operational costs

and increased productivity (Chen et al., 2007; Tomás-Pejó et al., 2009).

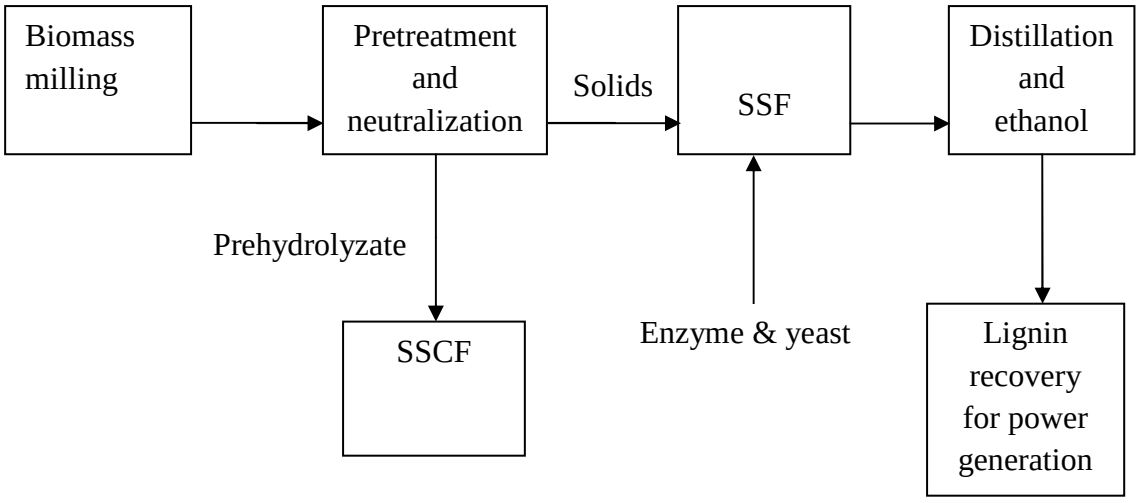

Fig.1.1 Schematic for production of ethanol from lignocellulosic feedstocks.

Cellulase enzymes used for hydrolyzing biomass have higher efficiency at elevated temperatures (Eriksen & Goksöyr, 1976; Ladisch et al., 1983; Ryu & Mandels, 1980). Hence, operating the SSF process closer to the optimum temperature of cellulase enzymes would be beneficial. Significant research has been done to identify thermotolerant microorganisms that can grow at high temperatures (Abdel-Fattah et al., 2000; Spindler et al., 1988; Szczodrak & Targonski, 1987; Yanase et al., 2010). Use of thermotolerant microorganisms would minimize operational costs with respect to maintaining growth temperature in reactors, decrease the chances of contamination, and facilitate the recovery of products (Singh et al., 1998). Hari Krishna et al. (2001) showed that *Kluyveromyces fragilis* NCIM 3358 performed better in SSF at 43°C and resulted in higher ethanol yields by producing 25 to 35 g L^{-1} than *Saccharomyces cerevisiae* NRRL-

Y-132 that only produced 20 to 25 g L^{-1} ethanol at 40°C.

Five thermotolerant yeast strains labeled IMB1, IMB2, IMB3, IMB4 and IMB5 identified as *K. marxianus* var. *marxianus* were isolated from a wine distillery in India (Banat et al., 1992). These strains were capable of fermenting glucose and other sugars at temperatures up to 52°C. *K. marxianus* IMB3 was capable of producing high concentrations of ethanol from cellobiose in the presence of externally added β-glucosidase and has a potential to be used in SSF (Singh et al., 1998).

Pretreated switchgrass (*Panicum virgatum*)as a source for cellulose was used in two earlier studies (Suryawati et al., 2009; Suryawati et al., 2008) in an SSF process using *K. marxianus* IMB4 with an external addition of cellulase enzyme (Fibrilase, Iogen, Ottawa, Canada) and resulted in production of 16.8 g L^{-1} ethanol (up to 78% of the maximum theoretical yield, MTY). The same research group compared the performance of IMB4 at 37, 41 and 45°C and obtained 12.3, 14.8, 15.8 g L^{-1} ethanol, respectively (Suryawati et al., 2008). It was also found that the increase in the pH of the SSF medium from 4.8 to 5.5 resulted in an increase in ethanol yields. In addition, *K. marxianus* IMB3 was capable of producing 19.5 g L^{-1} ethanol,corresponding to 80.7% of MTY, after 144 h of SSF with pretreated switchgrass at 45°C and Fibrilase enzyme with loading of 15 FPU g^{-1} glucan (Faga et al., 2010).

No reports on *K. marxianus* IMB3 in SSF of switchgrass using Accellerase 1500 enzyme (Genencor, Rochester, NY, USA) were reported in the open literature. According to the manufacturer, Accellerase 1500 is an enzyme mixture intended for production of ethanol from lignocellulosic biomass on industrial scale (Anonymous, 2009a). The

primary objective of this study is to investigate the effects of Accellerase 1500 loading, temperature and feeding strategies on ethanol production by *K. marxianus* IMB3 in SSFs with switchgrass.

CHAPTER II

LITERATURE REVIEW

2.1 Biomass feedstocks

2.1.1 Woody biomass

Woody biomass can be classified as softwoods and hardwoods. Softwoods are gymnosperms with needle-like leaves and are commonly referred to as evergreens. Pine and spruce are some examples of softwoods. Hardwoods are angiosperms that have broad leaves (Cheng, 2009). Poplar, willow and oak are some examples of hardwoods. The fast growing capacity of poplar makes it ideal for use in combustion, gasification and fuel production. Poplar can be grown on different soils as long as the pH of the soil is near 7 (Cheng, 2009). Hybrid poplar yields in North America are reported to be around 5 dry tons per acre. Hardwoods and softwoods contain 40% to 50% cellulose on a dry basis (Cheng, 2009). Hemicelluloses content of 11% to 20% is seen in softwoods, whereas hardwoods contain 15% to 20% hemicelluloses (Cheng, 2009; Galbe & Zacchi, 2002). Lignin content in softwoods is 27% to 30%, whereas hardwoods contain 20% to 25% lignin (Cheng, 2009; Galbe & Zacchi, 2002).

2.1.2 Agricultural residues

This type of biomass refers to either crop residues or processing residues. Corn

stover, rice straw, and wheat straw are some examples of agricultural residues, which are readily available for energy production. In the United States alone, around 500 million tons of agricultural residues are generated annually (Cheng, 2009).The typical compositions of these biomass materials are 35% to 40% cellulose, 17% to 35% hemicellulose and 7% to 18% lignin (Cheng, 2009). Among the three types of residues mentioned above, rice straw is the most abundant agricultural residue in the world with an annual global production of 731 million tons (Cheng, 2009).

2.1.3 Energy crops

2.1.3.1 Miscanthus

Miscanthus is a genus related to the sugarcane family and is found in a wide range of tropical and subtropical climates. Miscanthus can reach heights from 2m to 10m based on the location and can be harvested only once a year since multiple cutting results in the death of stands(Cheng, 2009). Miscanthus contains high cellulose (43%) and low ash content (less than 4%), which makes it suitable for combustion applications (Cheng, 2009).

2.1.3.2 Switchgrass

Switchgrass (*Panicum virgatum*) is a warm-season perennial grass that is native to North America is a C4 species capable of growing in prairies and marshes (McLaughlin et al., 1999). Due to its high biomass yield capacity, switchgrass was widely used earlier for forage purposes. “When managed for biomass production, switchgrass’ dense canopy and extensive network of roots can reduce raindrop impact, runoff and erosion” (Parrish & Fike, 2005).

Lowland and upland are the two ecotypes of switchgrass that occur. Lowland ecotypes (such as Kanlow and Alamo) are tall, thick stemmed and adapted to wet conditions (Cheng, 2009). Upland ecotypes (such as Cave-in-Rock and Trailblazer) are usually short, thin stemmed and adapted to drier conditions. Lemus et al. (2002) evaluated twenty varieties of switchgrass and found that Alamo and Kanlow switchgrass have the greatest biomass yields. Switchgrass biomass yields of up to 15 tons per acre have been reported in the United States (Thomason et al., 2005). Switchgrass's capability to prevent soil erosion, facilitate the breakdown of soil contaminants, and high water use efficiency make it an ideal choice as a feedstock for biofuel production (Lynd, 1996; McLaughlin et al., 1999).

In order to use switchgrass as an energy crop, one has to consider its cellulose, hemicellulose, and lignin content. The composition of switchgrass varies with each type. Switchgrass contains from 31% to 45% cellulose, 25% to 31% hemicelluloses, 10% to 17% lignin, 5% to 10% ash and 10% to 15% extractives (Alizadeh et al., 2005; Bals et al., 2010; Faga et al., 2010; Suryawati et al., 2009; Suryawati et al., 2008). Typically, carbohydrates and organic compounds constitute the cell wall of switchgrass. Structural carbohydrates in switchgrass include polymers of D-glucose, D-xylose, D-arabinose, D-galactose and D-mannose. The main structural material of the cell wall in switchgrass is cellulose, a linear homo-polysaccharide of D-glucose units linked via β-1,4-glycosidic bonds with a degree of polymerization of 10,000 or higher (Lynd, 1996). The crystallinity of cellulose is due to hydrogen bonding between cellulose chains that are typically arrayed in a parallel arrangement (Jørgensen et al., 2007; Lynd, 1996). Hemicellulose is a heterogeneous polysaccharide with a branched structure that is composed of D-glucose,

D-xylose, D-galactose, D-arabinose, D-mannose, D-galacturonic acid and 4-O-methyl-D-glucoronic acid (Lynd, 1996). In grasses, hemicelluloses are primarily composed of glucuronoarabinoxylans (Lynd, 1996). Lignin is a large polymer of phenylpropanoid units (Jørgensen et al., 2007). Cellulose is embedded by lignin, which protects it from chemical and microbial degradation. Also, lignin forms covalent bonds with some hemicelluloses, such as benzyl ester bonds with the carboxyl group of 4-O-methyl-D-glucoronic acid in hemicellulose (Jørgensen et al., 2007). With respect to ethanol production from lignocellulosic biomass, lignin is not used by the microorganism. However, the heating value of lignin can be used for production of other value added products and for energy generation in a biorefinery (Lynd, 1996).

2.2 Pretreatment of biomass

With the complex, heterogeneous structure and recalcitrance of lignocellulosic biomass to degradation, the accessibility to cellulose is limited. Enzymatic hydrolysis of un-pretreated biomass resulted in only 20% theoretical ethanol yields; whereas, with pretreated biomass more than 90% theoretical ethanol yields have been reported (Alizadeh et al., 2005; Alvira et al., 2010; Lynd, 1996; Mosier et al., 2005).

Limited pore size in the heterogeneous biomass matrix limits the accessibility of β-glycosidic bonds to cellulase enzymes. In addition, cellulose in biomass materials is closely associated with hemicelluloses and carbohydrate rich micro fibrils surrounded by lignin (Alvira et al., 2010; Mosier et al., 2005).Thus, for the utilization of lignocellulosic materials for enzymatic hydrolysis, overcoming both physical and chemical barriers is necessary (Alvira et al., 2010; Mosier et al., 2005; Mtui, 2009; Sousa et al., 2009). The

goal of any pretreatment process is to disrupt the crystalline structure of cellulose, solubilize hemicelluloses, remove or redistribute lignin, and minimizes loss of sugars (Jørgensen et al., 2007; Mosier et al., 2005). A schematic on the effect of pretreatment on biomass is shown in Fig. 2.1.

Pretreatment methods can be classified as physical, chemical, physico-chemical and biological. Grinding, size reduction, extrusion and milling are some examples of physical pretreatment techniques. Chemical pretreatment techniques employ acids, bases, solvents or the combination of these chemicals. Biological pretreatment methods use microorganisms, such as white rot, brown and soft-rot fungi. Various pretreatment techniques are briefly discussed in the next sections.

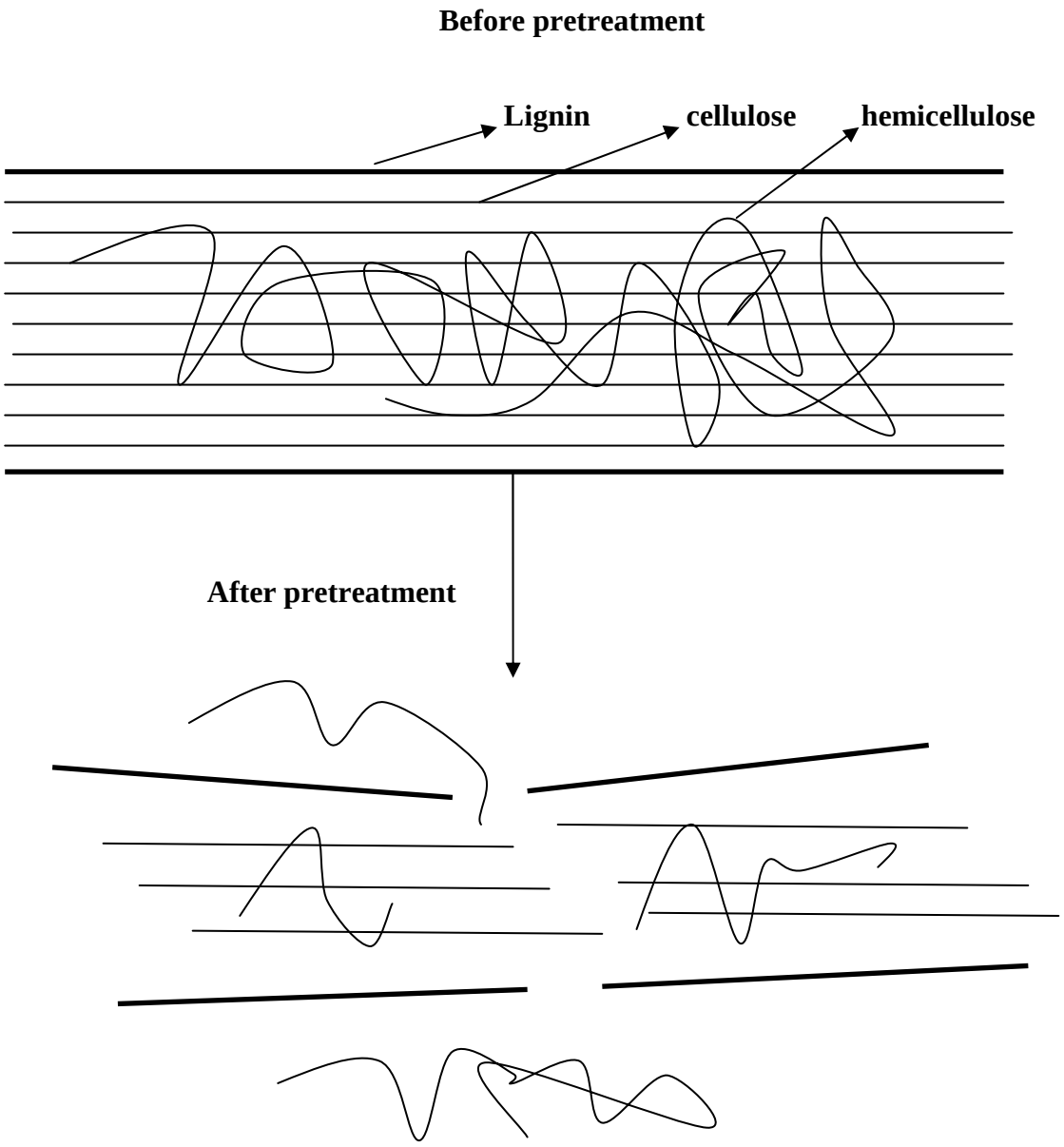

Fig. 2.1 Effect of pretreatment on biomass, adapted from (Mosier et al., 2005).

2.2.1 Ammonia based processes

Ammonia Fiber Explosion (AFEX) is a process in which biomass is treated with liquid ammonia at high temperature and pressure. A typical AFEX process is operated at 90°C with a residence time up to 30 min; however, the parameters vary depending on the

type of biomass feedstock used. AFEX decreases the crystallinity of cellulose and disrupts lignin structure (Bals et al., 2010; Kumar et al., 2009). In an AFEX process, 1 to 2 kg of ammonia per kg of dry biomass is used. The mixture of ammonia and biomass is heated to between 90°Cand 100°C with a residence time of 30 min. The pressure is released rapidly, which causes swelling and physical disruption of biomass fibers. Partial decrystallization of cellulose is also possible (Kumar et al., 2009; Lau et al., 2009). With an AFEX process, ammonia that is used can be recycled, which decreases the cost of the pretreatment process. The main advantage of AFEX is that no inhibitory compounds are formed with this pretreatment method (Bals et al., 2010; Lau et al., 2009; Sendich et al., 2008). AFEX has been widely used in recent years for the pretreatment of switchgrass. Two studies were reported on the optimization of AFEX for the pretreatment of switchgrass, which achieved more than 90% glucan conversion after enzymatic hydrolysis of the pretreated solids (Alizadeh et al., 2005; Bals et al., 2010).

Ammonia recycle percolation (ARP) is another method for pretreatment of biomass. In this method, aqueous ammonia is passed through a reactor packed with biomass. The temperature is maintained from 140°C to 210°C with a reaction time up to 90 min and a percolation rate of 5 mL min^{-1} (Sendich et al., 2008; Wyman et al., 2005). ARP solubilizes most of the hemicellulose while cellulose remains intact (Wyman et al., 2005).

2.2.2 Alkali pretreatments

Alkali pretreatments of biomass increase digestibility of cellulose and are effective for solubilization of lignin. Alkali pretreatments can be performed at ambient

temperatures with residence time varying from seconds to days (Alvira et al., 2010). Hydroxides of sodium, potassium, calcium and ammonium can be used for alkaline pretreatments. “Sodium hydroxide causes swelling of biomass, thereby increasing the internal surface area of cellulose, decreases the degree of polymerization and crystallinity of cellulose, which provokes the disruption of lignin structure” (Alvira et al., 2010). Calcium hydroxide, also known as lime, removes amorphous substances, such as lignin, and acetyl groups from hemicelluloses (Alvira et al., 2010; Kumar et al., 2009). The use of lime reduces the formation of inhibitory compounds and requires fewer safety precautions compared to NaOH or KOH (Alvira et al., 2010).

2.2.3 Dilute acid pretreatment

The main objective of dilute acid pretreatment is to dissolve hemicellulose and increase the accessibility of cellulose to enzymes (Mosier et al., 2005). Dilute acid pretreatment can be performed at high temperature (180°C) with a short residence time or at lower temperatures (120°C) for a longer period of time (Alvira et al., 2010; Mosier et al., 2005). Hydrochloric acid, phosphoric acid and nitric acid have been used in the past, but high hydrolysis rates have been reported when biomass is pretreated with dilute sulfuric acid (Alvira et al., 2010). In a dilute acid pretreatment method, acid catalyzes the breakdown of cellulose to glucose and further breakdown of glucose to form 5-hydroxymethylfurfural (HMF) and other degradation products (Kumar et al., 2009; Mosier et al., 2005). The typical acid concentrations used vary from 0.7% to 4%. Another type of acid pretreatment is the flow-through acid pretreatment in which very dilute sulfuric acid (0.07%) is added in a flow-through reactor configuration (Mosier et al., 2005). Temperatures ranging from 140°C to 200°C with a retention time ranging from 10

min to 20 min have been tested. This process removed about 83% to 100% of hemicellulose (Mosier et al., 2005).

2.2.4 Steam explosion

In this method, biomass is treated with high-pressure saturated steam followed by sudden release of pressure that makes biomass undergo an explosive decompression. During pretreatment, acetic acid and other acids are formed from the acetyl groups present on hemicellulose and hydrolyze hemicellulose (Alfani et al., 2000; Mosier et al., 2005). Steam explosion removes hemicellulose and improves the accessibility of enzymes to cellulose. Due to the explosive decompression in the pretreatment process, biomass undergoes fragmentation, thereby increasing the accessible surface area (Alvira et al., 2010; Mosier et al., 2005). Steam explosion with addition of a catalyst has been studied widely and has been claimed to be close to commercialization (Kumar et al., 2009). Sulfuric acid, sulfur dioxide or carbon dioxide can be added to the steam explosion reactor to decrease the pretreatment time, the formation of inhibitors and completely remove hemicellulose (Kumar et al., 2009).

2.2.5 Ozonolysis

Using the powerful oxidative property of ozone is the basis of ozonolysis pretreatment. Ozonolysis removes lignin without any loss of cellulose content (García-Cubero et al., 2009). Pretreatment with ozone does not form any inhibitors that interfere with hydrolysis and fermentation of biomass (García-Cubero et al., 2009). Besides the advantages of ozonolysis, the pretreatment process requires large amounts of ozone and is not economically viable (Sun & Cheng, 2002). However, the effect of ozonolysis

pretreated biomass on ethanol production has not been widely studied.

2.2.6 Hydrothermolysis

Hydrothermolysis, or liquid hot water pretreatments, use high pressure to maintain water in liquid state at high temperature. This method is operated between 190°C and 230°C for up to 15 min. Hydrothermolysis pretreatment solubilizes up to 90% hemicellulose, partially removes lignin and preserves most of the cellulose (Mosier et al., 2005). *O*-acetyl and uronic acid groups present on hemicellulose are cleaved during hydrothermolysis to generate acetic acid. The release of such acids catalyzes the removal of oligosaccharides (Mosier et al., 2005; Wyman et al., 2005). However, the hemicelluloses are further hydrolyzed to monomeric sugars, xylose and glucose, which are further partially converted to furfural, levulinic acid and HMF, respectively (Alvira et al., 2010; Mosier et al., 2005). The acidic property of water at higher temperatures (pH = 5 at 200°C) and its high dielectric constant contribute towards solubilizing hemicellulose (Mosier et al., 2005). Higher lignin solubilization is not possible in hydrothermolysis because lignin recondenses during cooling after pretreatment (Jørgensen et al., 2007).

Several reactor configurations, such as co-current and counter-current flow-through and batch, have been used (Mosier et al., 2005). Mok and Antal (1992) pretreated samples of six woody and four herbaceous biomass species using hot compressed liquid water in a flow through tubular percolating reactor and achieved up to 60% solubilization of solids. All of the hemicellulose was solubilized and 80% of cellulose was retained in the pretreated solids. Weil et al. (1998) used hot water at 220°C, 240°C and 260°C for

pretreatment of corn fiber at a loading of 4% solids. The holding time was less than 10 sec. Potassium hydroxide was added to maintain the pH above 5. Subsequent enzymatic hydrolysis of pretreated solids resulted in 84% cellulose conversion to glucose (Weil et al., 1998). In another study, Ingram et al.(2009) used a semi continuous fixed-bed reactor for hydrothermolysis of rye straw. The optimum temperature range was between 170°C and 210°C. Subsequent hydrolysis of pretreated biomass resulted in more than 90% conversion of cellulose to glucose (Ingram et al., 2009).

Suryawati et al. (2009) optimized the conditions for pretreatment of switchgrass using hydrothermolysis. Switchgrass was loaded at 10% solids (dry basis) and various temperatures and holding times were tested. It was found that all treatments produced less than 1 g L^{-1} of HMF and furfural. Acetic acid concentration increased from 2.0 to 3.4 g L^{-1} when the holding time was increased from 10 to 20 min at 190°C and from 3.4 g L^{-1} to 4.0 g L^{-1} with increase of holding time from 10 to 15 min at 210°C (Suryawati et al., 2009). However, no further increase in acetic acid concentration was noticed when the holding time was increased from 15 min to 20 min. Subsequent SSF of pretreated switchgrass obtained at various pretreatment conditions were evaluated. The highest cellulose to ethanol yields were obtained with switchgrass that was treated at 200°C for 10 min (Suryawati et al., 2009).

2.2.7 Other pretreatment methods

Other pretreatment methods include organosolvation, ionic liquids pretreatment, microwave pretreatment, oxidative delignification, pulsed electric field pretreatment and biological pretreatment (Alvira et al., 2010; Kumar et al., 2009).Summary of various

pretreatment technologies is shown in Table 2.1.

Table 2.1 Various methods used for pretreatment of biomass.

Pretreatment method	Decrystallizes cellulose	Removal of hemicellulose	Alters/removes lignin	Disadvantages
Steam explosion	No	Yes	Partial	Formation of inhibitors
Dilute acid	No	yes	Yes	Formation of inhibitors
Hydrothermolysis	No	Yes	partial	Formation of inhibitors
AFEX	Yes	Partial	Yes	High cost of ammonia
Ozonolysis	No	Partial	Yes	Expensive
Alkali	Partial	Partial	Yes	Low rate of hydrolysis
Biological	NA	Yes	Yes	Low rate of hydrolysis

2.3 Hydrolysis of biomass

2.3.1 Hydrolysis of biomass using acid

The use of acids, such as sulfuric, hydrochloric and phosphoric acids, is the oldest and best known method for the hydrolysis of cellulose. Hydrolysis of cellulose by sulfuric acid is the most common method that has been used. In general, acid hydrolysis can be classified into dilute acid hydrolysis and concentrated acid hydrolysis. In diluted acid hydrolysis, 0.5% to 15% (w/w) sulfuric acid is used to hydrolyze cellulosic material

under high temperature and pressure (Choi & Mathews, 1996; Farone & Cuzens, 1997). Due to the high temperatures used in acid hydrolysis, some of the obtained monomeric sugars, such as xylose and glucose, degrade to form furfural, levulinic acid and HMF (Choi & Mathews, 1996). Moreover, low yields of glucose from cellulose (<50%) have been reported using dilute acid hydrolysis (Farone & Cuzens, 1997). Concentrated sulfuric acid hydrolysis uses 60% to 90% (w/w) sulfuric acid for the hydrolysis of biomass. The use of concentrated sulfuric acid produced better glucose yields compared to dilute acid hydrolysis. However, the production of inhibitory compounds, cost of acids, problems with handling, use of expensive reactors and recycling of acid are disadvantages of the concentrated acid hydrolysis method (Choi & Mathews, 1996; Von Sivers & Zacchi, 1995).

2.3.2 Enzymatic hydrolysis of biomass

Microorganisms produce multiple enzymes to degrade cellulose into simple sugars such as glucose or xylose. These enzyme complexes are generally termed as cellulases. Microorganisms such as *Trichoderma reesei* and *Humicola insolens* can produce cellulases (Castellanos et al., 1995; Ryu & Mandels, 1980). Cellulases are divided into three categories; endoglucanases, exoglucanases and β-glucanases. Exoglucanases move progressively along the cellulose chain and attack reducing ends of cellulose units to produce cellobiose. Endoglucanases randomly attack β-1,4-glycosidic bonds of cellulose and produce cellobiose (Jørgensen et al., 2007). Cellobiose is a dimer of β-D-glucose and is hydrolyzed by β-D-glucosidase enzyme to two D-glucose units. The presence of hydrolysis products such as cellobiose and glucose greatly influence the hydrolysis of cellulose (Lu et al., 2006; Lynd & Lee, 1989). For this reason, enzyme

complexes that are rich in β-D-glucosidase were manufactured and found useful in efficiently hydrolyzing cellulose. Zhou et al. (2009) found that using an optimized mixture of seven enzyme complexes containing cellobiohydrolases, Cel7A, Cel6A, Cel6B;endoglucanases, Cel7B, Cel12A, Cel61A; and β-glucosidase released glucose from steam exploded corn stover over two times faster than the original crude mixture. Cellulases generally have their highest activity between 45°C and 50°C at pH of 4.5 to 5.0 (Sun & Cheng, 2002). However, the optimum temperature and pH is based on the source of enzymes. Compared to hydrolysis, fermentation is performed at mild conditions between 30°C and 38°C, which limits the activity of enzyme. The composition of biomass also plays an important role in the enzymatic hydrolysis process. It was reported that lignin, which shields the cellulose chains, adsorbs up to 70% of the total enzyme and negatively affects the activity of cellulases (Jørgensen et al., 2007).

2.4 Fermentation schemes

Process configurations for the production of ethanol from biomass vary on the scale in which they are integrated. Various schemes have been developed for production of ethanol from biomass.

2.4.1 Separate hydrolysis and fermentation (SHF)

Separate hydrolysis and fermentation is a process configuration in which four reactors are involved. In this process, cellulase production, enzymatic hydrolysis of cellulose, fermentation of six carbon (C6) sugars, and fermentation of five carbon (C5) sugars take place in separate reactors. Since hydrolysis and fermentation are carried out in separate reactors, optimum conditions in each reactor can be maintained (Lynd, 1996;

Wingren et al., 2003). However, SHF has various disadvantages such as product inhibition caused by the accumulation of sugars during the hydrolysis step, risk of contamination by microorganisms due to the sugars present in the hydrolyzate, and most importantly, the cost of equipment involved (Alfani et al., 2000; Tomás-Pejó et al., 2009).

2.4.2 Simultaneous saccharification and fermentation (SSF)

SSF is a process in which hydrolysis of cellulose and fermentation takes place in a single reactor. Unlike SHF, the risk of product inhibition is minimized as the sugars obtained in hydrolysis are simultaneously utilized by the microorganism to produce ethanol. SSF reduces both capital cost and risk of contamination since the glucose released is quickly utilized by the ethanol-producing microorganisms (Alfani et al., 2000; Lynd, 1996; Tomás-Pejó et al., 2009). SSF has been widely studied with various microorganisms. The use of *S. cerevisiae* in SSFs has been widely studied (Faga et al., 2010; Spindler et al., 1989a; Stenberg et al., 2000; Wingren et al., 2003). However, if SSF is performed with commonly used yeast such as *S. cerevisiae*, the temperature must be maintained between 30°C and 37°C. Operating SSF at these temperatures reduces the activity of cellulase enzymes, thereby decreasing the overall efficiency of the process (Abdel-Banat et al., 2010). Hence, the use of microorganisms capable of growing above 37°C will be beneficial to reduce the cost of cooling of pretreated biomass and risk of contamination by other microorganisms (Singh et al., 1998).

Simultaneous saccharification and co-fermentation (SSCF) is a process configuration in which hydrolysis of cellulose and fermentation of C5 and C6 sugars are

performed in a single reactor. Unlike SHF, SSCF does not require four reactors, which reduces the capital cost (Lynd & Lee, 1989). Several microorganisms capable of fermenting both C5 and C6 sugars have been developed for use in SSCF. However, SSCF has several drawbacks such as slow hydrolysis rate due to the difficulty to maintain optimum conditions for hydrolysis and fermentation because of the use of a single reactor (Lynd, 1996). Thermotolerant microorganisms capable of fermenting both pentoses and hexoses should be used in SSCF to obtain high ethanol yields.

2.4.3 Consolidated bioprocessing (CBP)

CBP is a process in which cellulase production, enzymatic hydrolysis and fermentation of C5 and C6 sugars take place in a single reactor (Lynd, 1996). Microorganisms capable of producing cellulolytic enzymes and utilizing hexoses and pentoses are being studied widely (Lynd et al., 2005; Van Zyl et al., 2007; Warnick et al., 2002). CBP eliminates the use of multiple bioreactors and has the potential to make the overall process economic.

2.4.3.1 SSF using thermotolerant microorganisms

Significant research has been done on use of thermotolerant yeast strains for ethanol production. A total of 58 yeast strains belonging to 12 genera were assayed for their ability to grow and ferment carbohydrates at 40°C, 43°C and 46°C (Szczodrak & Targonski, 1987). The assayed yeasts belong to the genera *Aureobasidium, Candida, Cryptococcus, Fabospora, Kloeckera, Kluyveromyces, Pachysolen, Pichia, Saccharomyces, Schizosaccharomyces, Trichosporon* and *Torulopsis* (Szczodrak & Targonski, 1987). It was found that *Fabospora fragilis* CCy51-1-1 performed the best

compared to other strains by producing 56 g L^{-1} and 35 g L^{-1} ethanol from 140 g L^{-1} glucose in less than 48h at 43°C and 46°C, respectively (Szczodrak & Targonski, 1987). Hari Krishna et al. (2001) compared the ability of a thermotolerant strain *Kluyveromyces fragilis* NCIM 3358 with *S. cerevisiae* NRRL-Y-132. Sugar cane leaves and *Antigonum leptopus* leaves were used as a substrate with cellulase from *Trichoderma reesei* and supplemented with β-glucosidase. It was found that *K. fragilis* performed better in SSF at 43°C producing 25 to 35 g L^{-1} ethanol compared to SSF with *S. cerevisiae* at 40°C that produced 20 to 25 g L^{-1} ethanol.

Spindler et al. (1989b)performed an SSF using Sigma-cell 50 cellulose as a substrate with *Candida lusitaniae, Candida brassicae, Candida acidothermophilum,* and *Saccharomyces uvarum* at 37ºC, 41ºC, and 43ºC. A cellulase loading of 13 IU g^{-1} substrate was used for the SSFs. It was found that with increase of temperature, cell viability decreased. In addition, *S. uvarum* did not grow at 43ºC. The conversion rate of cellulose to ethanol also decreased from 55% to 71% with the increase in temperature for all of the yeast strains used. A similar study was performed by Ballesteros et al. (1991) in which 27 strains of yeast that belonged to the genera *Candida, Saccharomyces* and *Kluyveromyces* were tested. *K. marxianus* and *K. fragilis* produced the greatest ethanol concentrations of 21.9 g L^{-1} and 20.8 g L^{-1}, respectively, after 48 in media containing 50 g L^{-1} glucose when incubated at 45°C. When Solka-floc cellulose was used in SSF at 42°C, both of those strains produced 50% of theoretical ethanol yield after 78 h (Ballesteros et al., 1991). Edgardo et al. (2008) screened eleven *S. cerevisiae* strains for their ability to grow and ferment glucose in the temperature range of 35°C to 45°C. It was found that only two strains, IR2 and IR2*, were able to grow at 42°C. When an SSF was

performed with IR2 at 40°C using Kraft pulp and organosolv-pretreated *Pinus radiata* chips, 62% and 73% theoretical ethanol was obtained after 72 h, respectively (Edgardo et al., 2008). In another study by Nonklang et al. (2008), *K. marxianus* DMKU3-1042 was found to be capable of growing at 49°C and producing ethanol from glucose at 45°C. The same strain was also capable of utilizing cellobiose, xylose, xylitol, arabinose, glycerol, and lactose. However, no work has been reported on using this strain in SSF.

2.4.3.2 Thermotolerant IMB strains

Five thermotolerant yeast strains capable of growing at 52°C were isolated in a distillery in India (Banat et al., 1992). The isolates were identified as *K. marxianus* that produced between 57 g $L^{-1}$and 72 g L^{-1} ethanol at 45°C and from 50 g L^{-1} to 55 g L^{-1} ethanol at 50°C when grown on 140 g L^{-1}glucose. These strains were named IMB1, IMB2, IMB3, IMB4, and IMB5. When grown n 140 g L^{-1} glucose at 40°C, the five strains produced 67, 64, 65, 65, 68 g L^{-1} ethanol, respectively. The highest ethanol concentration of 72 g L^{-1} was obtained with IMB2 followed by IMB5 producing 70 g L^{-1} ethanol from glucose at 45°C. Banat and Marchant (1995) found that all five strains grew on lactose, whey permeate, cellobiose and xylose at 45°C. These strains also produced up to 95 g L^{-1} ethanol. The production of ethanol was not affected until its concentration in the medium reached to 75 g L^{-1} (Banat & Marchant, 1995).

Singh et al. (1998) reported that IMB3 produced 60 to 72 g L^{-1} ethanol at 42^{o}C in 16 to 20 h compared to similar amounts produced by a distillery strain of *S. cerevisiae* in 22 to 26 h. Several researchers have studied IMB3 in SSFs of cellulosic materials (Boyle et al., 1997; Faga et al., 2010; Kourkoutas et al., 2002; Nilsson et al., 1995). When IMB3 was used in SSFs of pulverized barley straw at 45°C at solid loadings of 2.0, 4.0 and 6.0

% (w/v) and supplemented with 2% (v/v) cellulase, maximum ethanol concentrations of 2.0, 3.0 and 3.6 g L^{-1} were obtained, respectively (Boyle et al., 1997). When the pulverized straw was replaced by NaOH pretreated straw at the same solid loadings, ethanol concentrations increased to maximum of 3.9, 8.0, and 12.0 g L^{-1}, respectively (Boyle et al., 1997).

2.4.4 Effect of substrate and enzyme loadings on SSF

The amounts of substrate and enzyme used play a prominent role in the rate of SSF. Increasing enzyme loading could result in an increase in the hydrolysis rate, but at the same time it will increase the production cost of ethanol. Hence, optimization of enzyme loading is required prior to performing SSF. It is also required to choose a solid loading that gives the highest ethanol concentration and yield.

Stenberg et al. (2000) investigated the effect of substrate and cellulase concentration on SSF. Substrate concentrations between 2.0 and 10.0% (w/v) and enzyme concentrations of 5.0, 10.0, 21.0 and 32.0 FPU g^{-1} cellulose were tested. Pretreated spruce (*Piceaa beas*) was used as a substrate for SSFs. It was found that with the increase in the enzyme concentration from 5.0 and 32 FPU g^{-1}, the ethanol yield increased from 42% to 74% with 2% solids (w/v), from 54% to 82% with 5% solids (w/v), and from 53% to 73% with 7.5% solids (w/v), respectively (Stenberg et al., 2000). It was also found that with increase in solid loading, the time needed to reach a maximum ethanol concentration increased. However, no fermentation products were observed and glucose accumulated with 10.0% (w/v) solids. For each cellulose concentration tested, the highest ethanol yield was obtained with 5.0% (w/v) solids (Stenberg et al., 2000). In another study by Hari Krishna and Chowdary (2000), alkaline hydrogen peroxide ($NaOH + H_2O_2$) pretreated

Linn (*A. leptopus*) leaves were used as a substrate in an SSF at 35°C to 45°C and the effect of enzyme loading within the range of 25 to100 FPU g^{-1} of substrate and substrate loading within 5 to 15% (w/v) was tested. A cellulase mixture of Celluclast and Novozym 188 (Novozymes, Bagsvaerd, Denmark) was used. It was found that 100 FPU g^{-1} substrate gave the highest ethanol yields. It was noticed that within the range of 50 to 100 FPU g^{-1} substrate, increasing the solid loading resulted in an increasing ethanol yield.

Though increased ethanol yields can be obtained with increased solid content, the high solid content often leads to problems with mixing in SSF. Hence, solids could be added in a fed-batch mode to reduce the risks associated with mixing at high solid loadings.

Faga et al. (2010) used hydrothermolysis pretreated switchgrass as a substrate in SSF using *K. marxianus* IMB3. The SSF was conducted with substrate loading of 40 g glucan L^{-1} at 45°C and the effect of decreased cellulase loading was studied. The enzyme (Fibrilase, Iogen, Ottawa, ON, Canada) loading was decreased from 15 FPU g^{-1} glucan to 10 or 5 FPU g^{-1} glucan. It was found that with decreasing the enzyme loading, the hydrolysis rate and ethanol yield decreased. With 5 and 10 FPU g^{-1} glucan, the highest theoretical ethanol yields were 41.0% and 62.0% compared to 78.0% theoretical ethanol yield that was obtained with 15 FPU g^{-1} glucan.

Pryor and Nahar (2010) tested Accellerase 1000 (Genencor International, Rochester, NY, USA), Spezyme CP (Genencor), and Celluclast 1.5L (Novozymes, Inc., Bagsvaerd, Denmark) in hydrolysis and SSF of Sunburst switchgrass. The effect of different pretreatments on enzymatic hydrolysis of switchgrass was compared with each

enzyme tested. The authors conducted hydrolysis experiments at 2.0 % (w/v) substrate loading with an enzyme loading of 25 FPU g^{-1} substrate. Spezyme CP and Celluclast 1.5L were supplemented with Novozym 188, β-glucosidase, to get a total loading of 31.3 cellobiase units per mL (CBU mL^{-1}) of cellulose . When biomass from acid pretreatment, alkaline pretreatment, and acid pretreatment was used, Accellerase 1000 resulted in15%, 19% and 5% lower glucose yields, respectively, compared to yields obtained with other enzyme complexes used (Pryor & Nahar, 2010). The increase in Accellerase 1000 loadings from 15 to 20 FPU g^{-1} cellulose resulted in an increase in glucose yields. However, no significant differences in glucose yields were measured when the enzyme loading was increased from 20 to 30 FPU g^{-1} cellulose (Pryor & Nahar, 2010). This showed that saturation of enzyme occurred at Accellerase 1000 loading of 20 FPU g^{-1} cellulose. It was also evident from the study that higher loadings of Accellerase 1000 were required compared to the other commercial enzymes tested (Pryor & Nahar, 2010).

2.4.5 Effect of temperature on SSF

Temperature plays an important role in SSF. It is very important to operate SSF at temperatures close to the optimum temperature of the enzymes. However, this is not often possible due to the limited thermotolerance of the microorganism used for fermentation. Various studies have been reported on optimization of temperature for SSF (Hari Krishna & Chowdary, 2000; Lark et al., 1997; Suryawati et al., 2008).

Lark et al. (1997) used recycled paper sludge as a substrate in an SSF using *K. marxianus* ATCC 36907 with temperatures from 25°C to 43°C using a cellulase mixture Fibrilase (Iogen, Ottawa, Canada) at a loading of 8 FPU mL^{-1}. An initial substrate concentration of 8.9% (w/v) and initial yeast cell concentration of 5.4 g L^{-1} were used.

The results showed that after 40 h of fermentation, with the increase in temperature from 25°C to 43°C, there was an increase in ethanol concentrations from 10.0 g L^{-1} to 13.8 g L^{-1}. However, 38°C was chosen in order to maintain the yeast activity for prolonged period of time during SSF. It was also mentioned that using 38°C gave better ethanol yields compared to SSFs at higher temperatures due to the prolonged activity of yeasts.

Anderson et al. (1986) performed glucose fermentation at 25°C, 39°C and 47°C using *K. marxianus*. It was found that higher ethanol yields were obtained at 39°C. After 20 h of fermentation, 60 and 70 g L^{-1} ethanol was obtained with fermentations at 47°C and 39°C, respectively. Cell death occurred with higher temperatures after 20 h and was more rapid with fermentations at 47°C compared to 37°C.

Chen et al. (2007) used temperature cycling to improve ethanol yields in SSF using steam exploded wheat straw as a substrate and an enzyme loading of 10 FPU g^{-1} substrate. The temperature was changed periodically between 37°C and 42°C such that the incubation time at 37°C was longer than the doubling time of yeast and incubation time at 42°C was less than 20 min. The results showed that with temperature cycling 52 g L^{-1} ethanol was obtained, which was two times higher than that observed at 37°C without temperature cycling.

Suryawati et al. (2008) used hydrothermolysis pretreated switchgrass to study the effect of temperature on SSF using *K. marxianus* IMB4. SSFs were performed at 37°C, 41°C and 45°C with Fibrilase loading of 15 FPU g^{-1} glucan and a glucan loading of 4.1% (w/v). The results were compared to SSFs using *S. cerevisiae* D_5A at 37°C. It was found that SSFs at 45°C performed the best at 72 h compared to all other treatments. However,

no significant difference in ethanol yields were noticed with SSFs at 41°C compared to all other treatments. With SSFs at 37°C, no significant glucose accumulation was noticed after 96 h. However, with SSFs at 41°C and 45°C, glucose started accumulating after 96 h.

2.4.6 Effect of addition of media components and reinoculation on SSF

The use of optimized media is very important for efficient SSFs. During the fermentation process, nutrients are utilized by the microorganism used, which results in the depletion of certain nutrients. Sufficient nutrient supply should be added to the medium used in SSF to maintain the viability of cells.

Ballesteros et al. (1994) attempted to increase ethanol yields by supplementing SSF media initially with unsaturated fatty acids and sterols. The SSFs were performed at 42°C using *K. marxianus* EMS-26. It was found that addition of these compounds decreased hydrolysis rates and ethanol production as compared to controls with no addition of unsaturated fatty acids and sterols (Ballesteros et al., 1994). In another study, Ballesteros et al. (1998) determined the effect of surfactants and zeolite-like products (ZESEP-56 from sepiolite and ZECER-56 from ceramic residues) on SSF of steam-exploded poplar using *K. marxianus* EMS-26. It was found that addition of 0.4 g L^{-1} of Tween-80 increased the enzymatic hydrolysis yield by 20% compared to controls (with no addition of surfactants). It was also found that the addition of ZESEP-56 and ZECER-56 increased ethanol yields by 14% and 20%, respectively. The addition of zeolite also decreased the fermentation time to 10 h compared to 24 h without additives, which was due to the increase of pH caused by the ion exchange property of the zeolite-like products. The increase in ethanol yields were due to removal of inhibitors formed during

pretreatment by the zeolite-like products used (Ballesteros et al., 1998).

Gough et al. (1996) used molasses as a substrate for the production of ethanol using *K. marxianus* IMB3. The effect of magnesium, potassium, nitrogen and linseed oil was evaluated. It was found that magnesium and linseed oil had a positive effect on ethanol yield and productivity. Addition of magnesium sulfate and linseed oil to molasses increased ethanol productivity by fivefold (from 1.0 to 4.8 g L^{-1} h^{-1}) and addition of potassium increased ethanol concentration from 7.4 to 8.5% (v/v).

Suryawati et al. (2008) investigated the effect of media concentration on SSF at 45°C with pretreated switchgrass using *K. marxianus* IMB4. It was found that the increase in the concentrations of media components in SSF by threefold of the usual concentration used resulted in a decrease in ethanol yield from 78.0% to 56.9%.

2.4.7 Effect of increased solid loading and feeding strategies

For a lignocellulosic ethanol process to be industrially viable, ethanol concentrations must be more than 4% (v/v), which requires the operation of SSF process at high solid loadings (Hack & Marchant, 1998; Jørgensen et al., 2010). SSFs have been performed at high solid loadings in various studies (Hoyer et al., 2010; Jørgensen et al., 2010; Varga et al., 2004). Jørgensenet al. (2010) used palm kernel press cake (PKC) as a substrate in an SSF using *S. cerevisiae* at a solid loading of 35% (w/v) and obtained 200 g ethanol per kg PKC, which was equivalent to 70% of theoretical yield. The same research group found that mannases, β- mannosidases and cellulase mixtures hydrolyzed PKC without the requirement of a pretreatment step, which resulted in fivefold increase in glucose yields (Jørgensen et al., 2010). In another study, Varga et al.(2004) used acid and

alkali wet oxidized corn stover as feedstocks in an SSF at a solid loading of 12% (w/v) at 30 FPU g^{-1} dry matter using *S. cerevisiae*. About 52 g L^{-1} of ethanol was obtained after 120 h of SSF which was equivalent to 83% of ethanol yield. The authors found that the increase in solid loadings from 12% to 20% (w/v) using acidic wet oxidized corn stover decreased ethanol yields from 85% to 5.7%. It was also found that when alkaline wet oxidized corn stover was used in an SSF with solid loadings of 17% (w/v), ethanol yields decreased to 78% compared to 83% with 12% solids (Varga et al., 2004).

The presence of high solids in an SSF increases the viscosity of the fermentation broth, which poses difficulties in mixing and increases the power consumption of the reactors (Hack & Marchant, 1998). Moreover, it reduces the heat transfer efficiency and results in end product inhibition of cellulases by accumulation of glucose or xylose (Jørgensen et al., 2010; Rudolf et al., 2004; Varga et al., 2004). In order to reduce the problems caused by high solid loadings in SSF and improve ethanol yields, fed-batch strategy in which solids are added at different time intervals has been studied (Hoyer et al., 2010; Jørgensen et al., 2010; Rudolf et al., 2004; Varga et al., 2004).

Nilsson et al. (2001) used hydrolyzates from forest residues for ethanol production using *S. cerevisiae* in batch and fed-batch strategies. It was found that fed-batch fermentation facilitated a complete utilization of sugars compared to batch process, in which only 23% of sugars were utilized (Nilsson et al., 2001).

Olofsson et al. (2010)investigated the effects of enzyme and substrate (pretreated wheat straw) feeding strategy on xylose conversion during SSCF by *S. cerevisiae* TMB3400, which ferments xylose. The SSCF was started with 8% (w/v) solids with a

gradual increase in solid loading to 11% (w/v). When both substrate and enzyme were added at different time intervals during the SSCF process, the conversion of xylose was 50% compared to 40% with only feeding substrate (Olofsson et al., 2010). The effects of enzyme feeding strategy on ethanol yields was investigated in a fed-batch SSF of pretreated spruce at 10% (w/v) and 14% (w/v) solid loadings using *S. cerevisiae* with cellulase mixture (total cellulase activity was 5 FPU g^{-1} dry solids and β-glucosidase activity of 8 IU g^{-1} dry solids) (Hoyer et al., 2010). It was found that the ethanol yield in fed-batch SSF increased to 60% compared to 50% in batch mode. In addition, fed-batch SSFs resulted in better mixing compared to batch mode.

CHAPTER III

OBJECTIVES

The objectives of this study were:

1. To determine the enzyme loading that results in the highest ethanol concentration in simultaneous saccharification and fermentation (SSF) of hydrothermolysis-pretreated switchgrass using thermotolerant *K. marxianus* IMB3 at 45°C.
2. To investigate the effect of temperature on SSF of hydrothermolysis-pretreated switchgrass using thermotolerant *K. marxianus* IMB3.
3. To conduct SSF of hydrothermolysis-pretreated switchgrass at high solid loadings and investigate the effect of solid and enzyme feeding strategies on ethanol yields with *K. marxianus* IMB3 at 45°C

CHAPTER IV

MATERIALS AND METHODS

4.1 Sample preparation

Kanlow switchgrass (*Panicum virgatum*) was harvested from Oklahoma State University Plant and Soil Sciences research center and milled through a 13 mm screen. Prior to compositional analysis, switchgrass was ground through a 2 mm screen using a Thomas-Wiley mill (Model 4, Arthur H. Thomas Co., Philadelphia, PA, USA). National Renewable Energy Laboratory (NREL) procedures were used to perform compositional analysis (Sluiter et al., 2008; Sluiter et al., 2005; Sluiter et al., 2004a). Acid soluble lignin was measured at 205 nm using a UV-Vis spectrophotometer (Cary 50 bio, Varian Inc., Palo Alto, CA, USA). The suggested 205 mm wavelength was chosen based on work done by Thammasouk (1997). A two-step extraction process was performed using an NREL procedure (Sluiter et al., 2005) prior to determination of structural carbohydrates and lignin in biomass. Automatic extraction by ethanol followed by water was conducted using an ASE® 300 system (Dionex Corporation, Sunnyvale, CA, USA). The operating parameters for both steps were 1,500 psi at 100^{o}C, 150% flush volume, 7 min static time, 2 min purge time, and 3 static cycles. All extractions were done in triplicate in 33 mL extraction cells using 95% ethanol and distilled water for ethanol and water extractions, respectively. Removal of solvents from extractives was done using a Rapidvap® N_2

evaporation system (Labconco Corporation, Kansas City, MO, USA) set at 500 mbar and 40°C until all solvents were evaporated. Extracted switchgrass solids were air dried for at least 24 h prior to use in subsequent analysis of structural carbohydrates and lignin.

4. 2 Hydrothermolysis

Hydrothermolysis of switchgrass was conducted in a 1-L bench top pressure reactor (Parr series 4520, Parr instrument company, Moline, IL, USA) equipped with a propeller agitator, a 1 kW electric resistance heater and a temperature controller (Fig. 4.1). Switchgrass harvested in November 2009 after a freeze was used in this study. The reactor was filled with 60 g of switchgrass (dry basis) and 540 g of DI water to achieve a 10.0% dry matter mixture. The agitator was set at 500 rpm and the desired temperature was set to 200°C. After 200°C was reached, the sample was held at 200°C for 10 min. After pretreatment the reactor vessel was cooled down to 40°C using an ice bath. Subsequently, the contents of the reactor were separated into solid and liquid fractions by vacuum filtration using a Buchner funnel lined with Whatman filter paper #5 (Whatman PLC, Brentford, UK). The obtained prehydrolyzate was stored at 4°C for analysis of sugars. The solids were washed repeatedly with 2 L of warm water (60°C) to remove any residual sugars or inhibitors. The solids were then stored in plastic bags at 4°C until they were ready for use. Structural carbohydrates in pretreated switchgrass were quantified and determined according to NREL procedure (Sluiter et al., 2004a) using HPLC equipped with Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) for detecting organic acids and furfurals and HPX-87P (Bio-Rad, Hercules, CA, USA) column to detect sugars, with refractive index detection (1100 series, Agilent, Santa Clara, CA, USA) (Sluiter et al., 2004a; Sluiter et al., 2004b).

Fig. 4.1 PARR reactor.

4.3 Determination of cellulase activity

Accellerase 1500 was used in all SSF experiments, which is a commercially available enzyme made by Genencor (Rochester, NY, USA). The procedure to determine cellulase activity used was the standard filter paper assay (Ghose, 1987). The substrate used was 50 mg Whatman #1 filter paper strip (1×6 cm), which was rolled and placed into 13×100 mm test tubes. The strips were immersed in 1.0 mL of 0.05M Na-citrate buffer at a pH of 5.0. Four dilutions were made such that at least one dilution releases 2.0 mg of glucose. Two types of controls were used in the assay: (a) enzyme control for each dilution (1.0 mL 0.05 M Na-citrate buffer + 0.5 mL enzyme dilution) and (b) substrate control (1.5 mL 0.05 M Na-citrate buffer + filter paper strip) and a reagent blank (1.5 mL citrate buffer). The tubes with buffer solution and substrate were equilibrated at 50°C.

Then, 0.5 mL of diluted enzyme was added to the tubes. Following incubation for 60 min at 50ºC, 3.0 mL dinitrosalicylic acid (DNS) reagent was added to stop hydrolysis and combine with reducing sugars to provide a colorimetric indicator of glucose concentration. The tubes were then boiled in a water bath for 5 min and subsequently transferred into an ice bath. The absorbance of each enzyme concentration was measured at 540 nm on a UV-Vis spectrophotometer (Cary 50 Bio, Varian Inc., U.S.A). A calibration curve of glucose concentration versus absorbance was created with stock solutions of glucose at different concentrations (Ghose, 1987). From the standard glucose curve, the amount of glucose released for each sample tube was determined. Glucose calibration curve and other calculations related to determining enzyme activity are found in Appendix B. The amount of glucose released by each enzyme concentration was then used to determine the activity of the cellulase in filter paper units per mL of enzyme (FPU mL^{-1}).

$$Enzyme\ Activity\ (\text{FPU mL}^{-1}) = \frac{0.37}{[enzyme]_{release\ 2.0\ mg\ glucose}}$$

Where [enzyme] represents the proportion of original enzyme solution present in the directly tested enzyme dilution.

4.4 Microorganism and inoculum preparation

Cultures of *K. marxianus* IMB3 and *S. cerevisiae* D_5A were grown on liquid yeast extract peptone dextrose (YPD) medium containing: yeast extract 10 g L^{-1}, peptone 20 g L^{-1} and glucose 50 g L^{-1}. A loopful of IMB3 and D_5A cultures was aseptically transferred into 250 mL baffled culture flasks containing 100 mL of YPD medium and the flasks were covered with aerobic stoppers (Bug stopper, Whatman PLC, Florham

Park, NJ, USA). The IMB3 and D_5A inoculum were incubated at 45°C and 37°C, respectively, for 16 h at 250 rpm on an orbital shaker (MaxQ 4450, Thermo Scientific, Dubuque, IA, USA).The cells were centrifuged at 3,500 rpm for 7 min, the supernatant was decanted and cells were washed twice with DI water. The obtained cells were resuspended in DI water to give an OD of 56 for IMB3 and an OD of 50 for D_5A. One mL of these concentrated cells was used in SSF flasks to obtain an initial OD value of 0.56 and 0.5 for IMB3 and D_5A respectively. The concentration of the cells was 20 g L^{-1} for both IMB3 and D_5A.

4.5 Simultaneous Saccharification and Fermentation (SSF)

The medium used in all SSFs was a yeast fermentation medium (YFM), which was prepared by adding 5 g of yeast extract, 20 g KH_2PO_4, 10 g of $MgSO_4.7H_2O$, 20 g of $(NH_4)_2SO_4$, and 1 g of $MnSO_4.H_2O$ to 1 L of DI water (Banat et al., 1992). Commercially available cellulase, Accellerase 1500 (Genencor, Rochester, NY, USA), with an activity of 82.2 FPU mL^{-1} was used for all SSFs. Cellulase activity was determined using a standard filter paper assay (Ghose, 1987) as shown in Appendix B.

4.6 Effect of enzyme loadings on SSF

In order to determine the optimum enzyme loading required in SSF, three enzyme loadings, 0.3, 0.5 and 0.7 mL g^{-1} glucan, were tested. SSF with each loading was performed in triplicate in 250 mL baffled flasks sealed with a rubber stopper fitted with a 1 way air valve (Check valve, Fisher Scientific, Pittsburgh, PA) to maintain an anaerobic environment. Each fermentation flask contained 10 mL of YFM, 5 mL 1M sodium citrate buffer at pH 5.5 and 8% solids (w/v), 1 mL of concentrated IMB3 culture or D_5A to give

a final cell concentration of 0.2 g L^{-1}. The total mass in each flask was 100 g. The pH of the medium was adjusted to 5.2 using 2N KOH. All flasks were incubated at 45°C on an incubating orbital shaker (MaxQ mini 4450, Thermo Scientific, Dubuque, IA). Samples were collected at 0, 6, 24, 48, 72, 96, 120, 144 and 168 h. The samples were centrifuged at 13,500 rpm for 10 min and the supernatant was frozen for later analysis. A control flask was maintained at each enzyme loading at 45°C, which contained the same media composition, excluding switchgrass. A sample calculation for ethanol yield and other parameters for SSF is shown in appendix B.

4.7 Effect of temperature on SSF

Using the same procedure described above, SSFs were performed at 37, 41 and 45°C in triplicate with the enzyme loading that gave the maximum ethanol yield from the previous experiment. The flasks were loaded with 8% solids (dry basis). Flasks with D_5A were incubated at 37°C, while flasks with IMB3 were incubated at 37, 41 and 45°C on an orbital shaker (MaxQ mini 4450, Thermo Scientific, Dubuque, IA) at 130 rpm. A control flask inoculated with D_5A and IMB3 was maintained at each temperature, which contained the same medium excluding switchgrass.

4.8. Effect of solid loading and feeding strategy

Using the optimum enzyme loading and temperature obtained from previous experiments, another set of experiments was performed in order to determine the effect of increased solid loading using fed-batch SSF. The media and cell concentrations were the same as in the previous experiments. The experiment contained four treatments (labeled as A, B, C and D). In treatment A, the SSF was performed with 12% solids with an enzyme loading of 0.7 mL g^{-1} glucan (i.e., total enzyme added initially was 4.85 mL

corresponding to 12% solids). In treatment B, the SSF was started with 8% solids with an enzyme loading of 0.7 mL g^{-1} glucan corresponding to 12% solids (i.e., 4.85 mL of enzyme was initially added). After 12 h, 4% solids were aseptically added. In treatment C, the SSF was started with 8% solids and 2/3 of the enzyme required for 12% solids (i.e., 3.23 mL of enzyme was initially added). After 12 h, 4% solids and 1/3 of the required enzyme (i.e., 1.62 mL of enzyme) were aseptically added. In treatment D, the SSF was started with 8% solids and an enzyme loading of 0.7 mL g^{-1} glucan corresponding to 8% solids (i.e., 3.23 mL of enzyme was initially added). After 12 h, 4% solids were added without addition of enzyme. All flasks were incubated at 45°C.

4.9 Sample analysis using HPLC

For analysis of acetic acid, succinic acid, xylitol, glycerol and ethanol from SSF samples, an Aminex HPX-87H column (Bio-Rad, Sunnyvale, CA, USA) maintained at 60°C was used with 0.01N H_2SO_4 as eluent flowing at 0.6 mL min^{-1}. For quantification of cellobiose, glucose, xylose, galactose and arabinose, an Aminex HPX-87P column (Bio-Rad, Sunnyvale, CA, USA) operated at 85°C with DI water as eluent flowing at 0.6 mL min^{-1} was used. For both columns, refractive index detection (1100 series, Agilent, Santa Clara, CA, USA) was used. Since the enzyme itself has some residual sugars present in it, ethanol concentrations obtained from the control flasks were subtracted from the concentration obtained from each SSF. Theoretical yield of ethanol production was calculated as follows:

$$\%\ theoretical\ yield = \frac{[EtOH]_t - [EtOH]_o}{0.511 \times (f[biomass] \times 1.11)} \times 100\%$$

Where $[EtOH]_o$ and $[EtOH]_t$ are the concentrations of ethanol at time 0 h and time t,

respectively. The parameter *f* is the glucan fraction of dry biomass and [biomass] is the dry biomass concentration. The factor (1.11) is the conversion factor for glucan to glucose.

4.10 Mass balance calculation

Mass balances were performed on glucose, xylose and lignin for the hydrothermolysis pretreatment and SSF experiments. For all calculations, glucose was assumed as the sole carbon source for the production of ethanol, acetic acid, glycerol and succinic acid. Glucose accounted for products was calculated as described in Faga et al. (2010). The conversion efficiency of glucan using Accellerase 1500 in all SSF experiments and the fate of lignin at the end of each SSF was determined by measuring the composition of solids at the beginning and end of each SSF using acid hydrolysis test (Sluiter et al., 2004a). Xylan balance was calculated by considering the amount of xylitol formed and the residual xylose that was present in the flasks at the end of each SSF. For all calculations, it was assumed that 60% of the solids were dissolved by the end of each SSF and 20% of the solids were removed from each SSF due to sampling.

4.11 Statistical analysis

A repeated measurements design was used to test the effects of temperature, enzyme loading and fed-batch strategy on ethanol yield using the GLM procedure in SAS statistical software (Release 9.2, Cary, NC, USA). Means were separated by Fisher's protected least significant difference test with a 95% confidence level.

CHAPTER V

RESULTS AND DISCUSSION

5.1 Composition of switchgrass and prehydrolyzate

Composition of switchgrass before and after pretreatment is listed in Table 5.1. The pretreated solids contained 57.7% glucan, 5.0% xylan and 35.1% lignin. The dissolved sugars were further hydrolyzed and converted to furfural and other products. The prehydrolyzate from the pretreatment contained 3.4 g L^{-1} glucose, 15.2 g L^{-1} of xylose, 3.4 g L^{-1} acetic acid and 3.8 g L^{-1} of furfural. Hydrothermolysis pretreatment removed about 15.8% glucan and 87.7% xylan from the switchgrass solids into the prehydrolyzate.

Table 5.1 Composition of switchgrass used in SSFs with *K. marxianus* IMB3 before and after pretreatment.

Compound	Composition prior to pretreatment (% db[a])	Composition after pretreatment (% db[a])
Glucan	41.9	57.7
Xylan	25.1	5.0
Galactan	0.7	0.0
Arabinan	2.2	0.0
Lignin	21.0	35.1
Extractives	5.5	ND[b]

[a]db = dry basis; [b] ND = not determined

5.2 Effect of enzyme loading on SSF

The increase in Accellerase 1500 (Genencor, Rochester, NY, USA) loading from 0.3 to 0.7 mL g^{-1} glucan during SSF with *K. marxianus* IMB3 at 45^{o}C showed an enhancement in glucan hydrolysis in the first 6 h (Fig. 5.1). Hydrolysis of glucan to glucose occurred faster than its fermentation in the first 6 h in all treatments, which explains glucose accumulation in the medium. All the glucose that was released during the hydrolysis of glucan was fermented by IMB3 during SSF from 24 to 96 h. Then, glucose started to accumulate in the medium due to the reduction of IMB3 activity. About 2.3 g L^{-1} glucose was accumulated by the end of the SSF (Fig. 5.1). This was similar to the results obtained by Faga et al. (2010) in which SSF of pretreated switchgrass resulted in glucose accumulation after 48 h with *K. marxianus* strains IMB4 and IMB5, after 72 h with both IMB1 and IMB2 and after 96 h with IMB3. The reduction in IMB3 fermentation ability after 96 h could be due to multiple stresses on cells such as high concentrations of ethanol and acetic acid in the medium.

Ethanol concentrations significantly increased ($p < 0.05$) with an increase in enzyme loading from 0.3 to 0.7 mL g^{-1} glucan (Fig. 5.1). The highest ethanol concentration of 22.3 g L^{-1} was obtained with SSFs using 0.7 mL g^{-1} glucan at 72 h, which was equivalent to 85% maximum theoretical yield (MTY) (Fig. 5.2). Ethanol concentration of 19.2 g L^{-1} (corresponding to 74% MTY) was obtained after 120 h with the enzyme loading of 0.3 mL g^{-1} glucan, which was 8% and 14% lower than the ethanol produced with 0.5 and 0.7 mL g^{-1} glucan, respectively. Ethanol concentrations in the control flasks were less than 0.1 g L^{-1}, which showed that there was little residual glucose or other fermentable sugars present in the crude enzyme used. Increasing the enzyme

loading above 0.7 mL g^{-1} glucan with similar SSFs with switchgrass did not increase ethanol yield (as shown in appendix A). Therefore, an enzyme loading of 0.7 mL g^{-1} glucan (57.5 FPU g^{-1} glucan) was selected for subsequent tests.

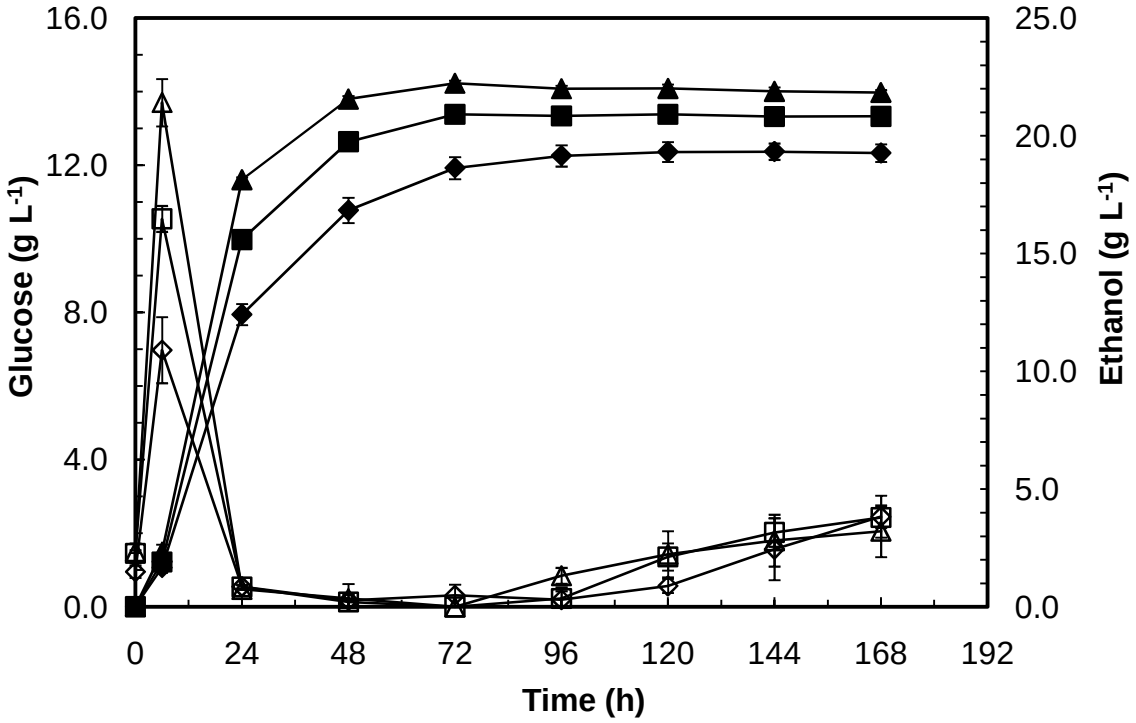

Fig.5. 1 Glucose (open symbols) and ethanol (solid symbols) profiles using *K. marxianus* IMB3 and 8% pretreated switchgrass in SSFs at 45°C and different enzyme loadings (mL g^{-1} glucan): (◊) 0.3, (□) 0.5, (Δ) 0.7 (n=3).

The use of high enzyme loadings increases the overall cost of ethanol production from lignocellulosic biomass. Moreover, there is a fair probability of saturation of the enzyme with the fixed amount of substrate added (Spindler et al., 1989a; Spindler et al., 1989b). Spindler et al. (1988) investigated the effect of enzyme loading on SSFs in the range of 7 to 21 FPU g^{-1} substrate and found that saturation occurred above an enzyme loading of 20 FPU g^{-1} substrate and saturation of enzyme occurred with high

β-glucosidase to cellulase ratios.

In another study, the effect of commercial cellulases (Celluclast 1.5L FG) on growth and ethanol production with glucose medium using *K. marxianus* CECT 10875 was investigated (Tomás-Pejó et al., 2009). It was found that high enzyme amounts (2.5 to 3.5 FPU g^{-1}) caused a negative effect on *K. marxianus* CECT 10875 growth and viable cell number. It was reported that additives that were present in the enzyme, such as sorbitol or glycerol, could have caused this effect (Tomás-Pejó et al., 2009).

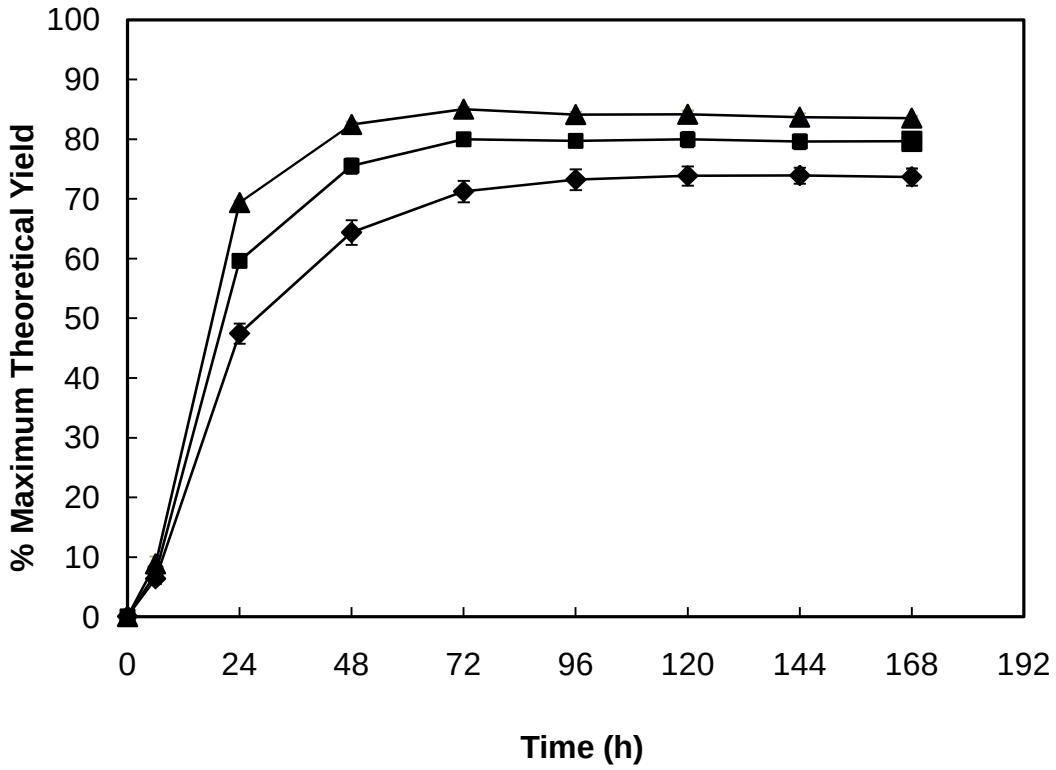

Fig. 5.2 Percentage of maximum ethanol theoretical yield using *K. marxianus* IMB3 and 8% pretreated switchgrass in SSFs at 45°C and different enzyme loadings (mL g^{-1} glucan): (♦) 0.3, (■) 0.5, (▲) 0.7 (n=3).

Acetic acid was produced during SSF with all enzyme loadings (Fig. 5.3). The final acetic acid concentrations were decreased as enzyme loading increased. Acetic acid production occurred at a constant rate for all enzyme loadings until 96 h. However after 96 h, the rate of acetic acid production remained constant with the enzyme loading of 0.3 mL g^{-1} glucan, while acetic acid production rate decreased with the other two enzyme loadings. SSFs with enzyme loading of 0.3 mL g^{-1} glucan produced 4.2 g L^{-1} acetic acid after 168 h, which was 11% and 15% higher than with 0.5 and 0.7 mL g^{-1} glucan, respectively.

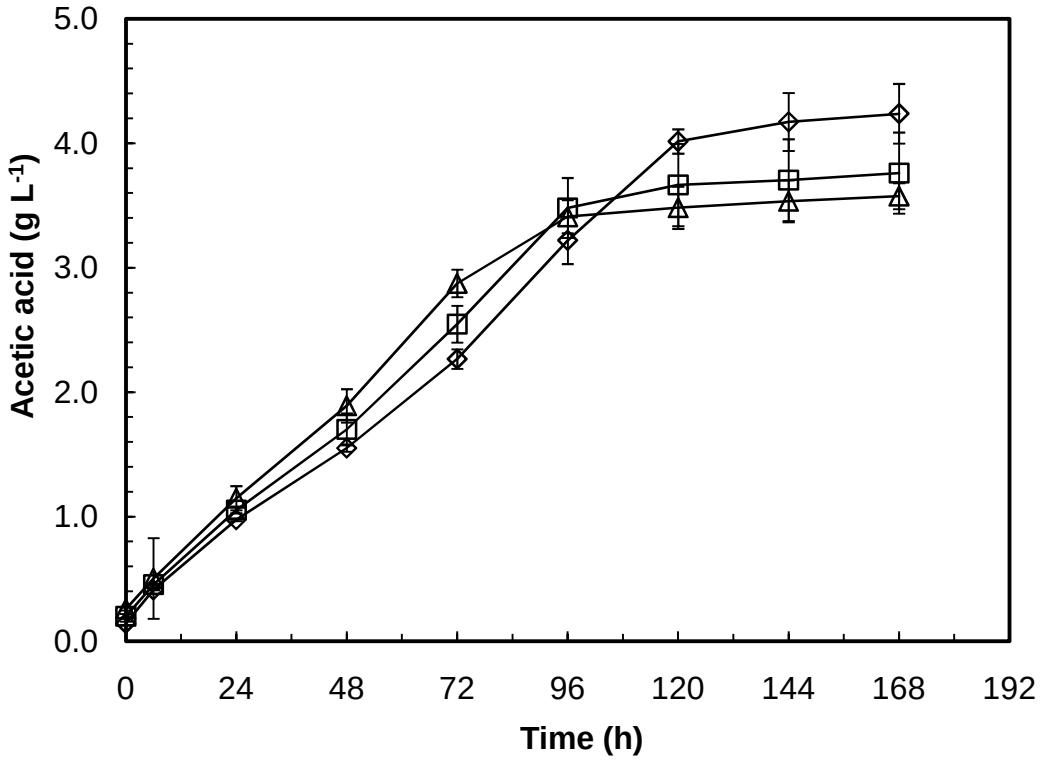

Fig. 5.3 Acetic acid profiles using *K. marxianus* IMB3 and 8% pretreated switchgrass in SSFs at 45°C and different enzyme loadings (mL g^{-1} glucan): (◊) 0.3, (□) 0.5, (Δ) 0.7 (n=3).

The final acetic acid concentration in SSF with enzyme loading of 0.3 mL g^{-1} glucan was significantly higher than with 0.7 mL g^{-1} glucan ($p < 0.05$). However, there were no significant differences in the amounts of acetic acid produced after 72 h of SSFs with enzyme loadings of 0.3 and 0.5 mL g^{-1} glucan or with 0.5 and 0.7 mL g^{-1} glucan ($p > 0.05$).

Other byproducts such as xylitol, glycerol and succinic acid were formed during SSFs with IMB3. IMB3 was found to produce xylitol from xylose (Mueller, 2009). The pretreated switchgrass contained 5% (db) xylan. Xylitol production increased from 0.8 g L^{-1} to 1.2 g L^{-1} after 168 h with an increase in the enzyme loading from 0.3 to 0.7 mL g^{-1} glucan. Glycerol production increased from 2.6 g L^{-1} to 3.4 g L^{-1} after 168 h, with an increase in enzyme loading from 0.3 to 0.7 mL g^{-1} glucan. SSFs with the three enzyme loadings resulted in approximately 0.7 g L^{-1} to 0.8 g L^{-1} of succinic acid after 168 h.

5.3 Effect of temperature on SSF

Accellerase 1500 at a loading of 0.7 mL g^{-1} glucan was chosen as the optimum enzyme loading to study the effect of temperature on SSF with *K. marxianus* IMB3. Saccharification of glucan during SSFs at the three temperatures (37, 41 and 45°C) occurred faster than ethanol production in the first 6 h, indicating the adaptive phase for IMB3 (Fig. 5.4). SSFs at 37°C had the lowest glucose concentration after 6 h, showing lower hydrolysis rates compared to 41°C and 45°C. Glucose concentrations decreased to less than 0.05 g L^{-1} at 24 h, due to the utilization of glucose by IMB3 to produce ethanol. Negligible concentrations of glucose were measured in SSFs with IMB3 at 37°C and 41°C. However, about 1.4 g L^{-1} glucose accumulated in SSFs at 45°C, after 96 h (Fig. 5.4). This was similar to the results obtained by Suryawati et al. (2008).

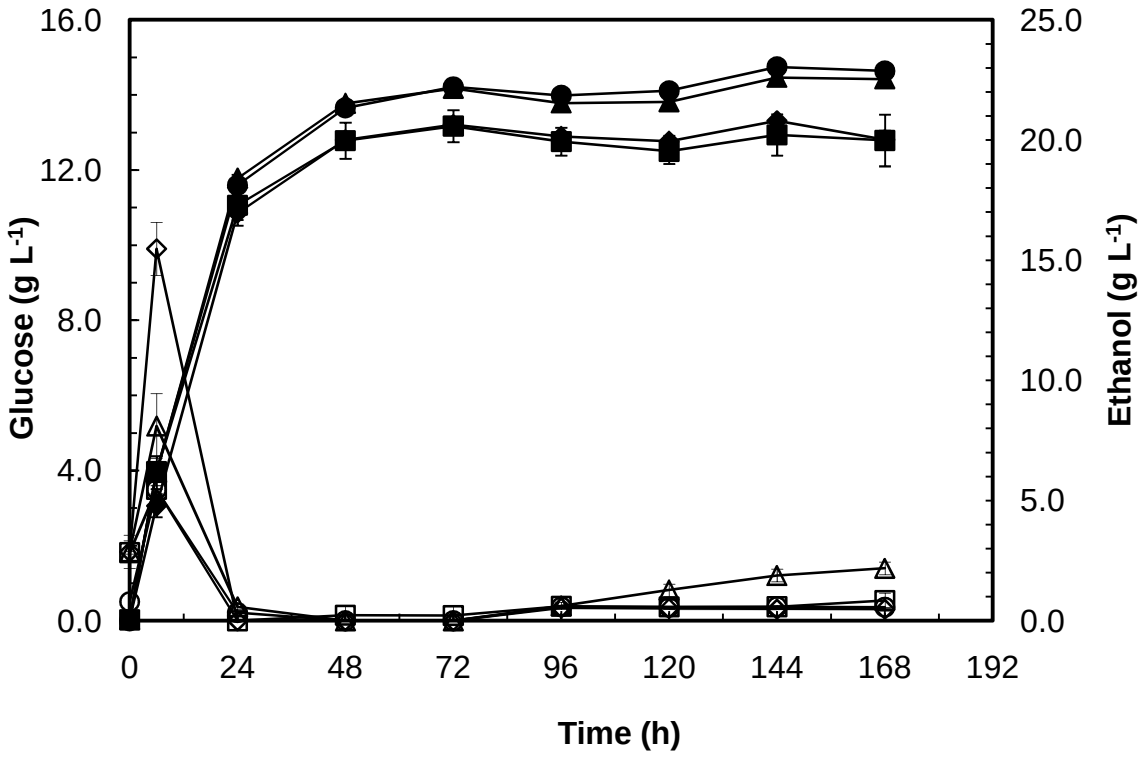

Fig. 5.4 Glucose (open symbols) and ethanol (solid symbols) profiles with *K. marxianus* IMB3 with 8% pretreated switchgrass and Accellerase 1500 at 0.7 mL g^{-1} glucan at different temperatures: (◊) 37°C, (□) 41°C, (Δ) 45°C, (○) 37°C with *S. cerevisia*e D5A (n=3).

SSFs at 45°C accumulated 1.4 g L^{-1} of glucose after 168 h, which was over 2.5 times higher than SSFs at 37°C and 41°C that accumulated 0.3 g L^{-1} and 0.5 g L^{-1}, respectively. Suryawati et al. (2008) and Faga et al. (2010) reported that *S. cerevisiae* D_5A performed better than IMB3 or IMB4 by achieving theoretical ethanol yields above 90% in SSFs with switchgrass and Fibrilase. For this reason, SSFs were performed in the present study at 37°C using *S. cerevisiae* D_5A and Accellerase 1500 at loading of 0.7 mL g^{-1} glucan.

SSFs at 37°C with *S. cerevisiae* D_5A resulted in negligible glucose accumulation after 96 h similar to IMB3 at 37 °C and 41°C (Fig. 5.4). Generally, there were no

significant differences in the concentrations of glucose obtained in SSFs with IMB3 and D_5A between 24 h and 96 h ($p > 0.05$). In addition, the differences in glucose concentrations during SSFs from 48 h to 168 h with IMB3 at 37 °C and 41°C and D_5A at 37 °C were insignificant ($p > 0.05$). However, the amounts of glucose accumulated during SSFs from 120 h to 168 h with IMB3 at 37 °C and 41°C and D_5A at 37 °C were lower than with IMB3 at 45°C ($p < 0.05$).

Ethanol production increased with time in all SSFs with IMB3 and D_5A (Fig.5.4). More ethanol was produced with IMB3 at 45°C and with D_5A at 37°C. The highest ethanol concentration (23.0 g L^{-1}) with IMB3 was obtained in SSFs at 45°C and 144 h ($p < 0.05$). No significant differences in ethanol production were measured in SSFs using IMB3 at 45°C or D_5A at 37°C ($p > 0.05$). In addition, insignificant differences in ethanol concentrations were measured in SSFs with IMB3 at 37°C and 41°C ($p > 0.05$).

Ethanol yields in all SSFs with IMB3 and D_5A increased substantially in the first 48 h, after which small increases in ethanol yields were measured (Fig. 5.5). Ethanol yields after 72 h of SSFs with IMB3 were 78.7 %, 78.5%, and 84.5% of MTY at 37, 41 and 45°C, respectively. The maximum ethanol yield (86.3%) with IMB3 was obtained in SSFs at 45°C after 144 h. Only 2% higher ethanol yield was obtained with D_5A at 37°C compared to IMB3 at 45^{o}C. One of the advantages in using IMB3 in SSFs using Accellerase 1500 compared to *S. cerevisiae* D_5A is that unlike D_5A, IMB3 is a thermophile that produces ethanol at temperatures above 37°C. Operation of SSFs at temperatures in the thermophilic zone reduces the possibility of contamination by mesophilic microorganisms and also enhances hydrolytic enzyme activities at temperatures close to their optimum values (Singh et al., 1998; Yanase et al., 2010). It

was reported that similar ethanol yields (approximately 70%) were obtained after 72 h in SSFs with *K. marxianus* IMB4 and Fibrilase loading of 15 FPU g^{-1} glucan at 41°C and 45°C (Suryawati et al., 2008).

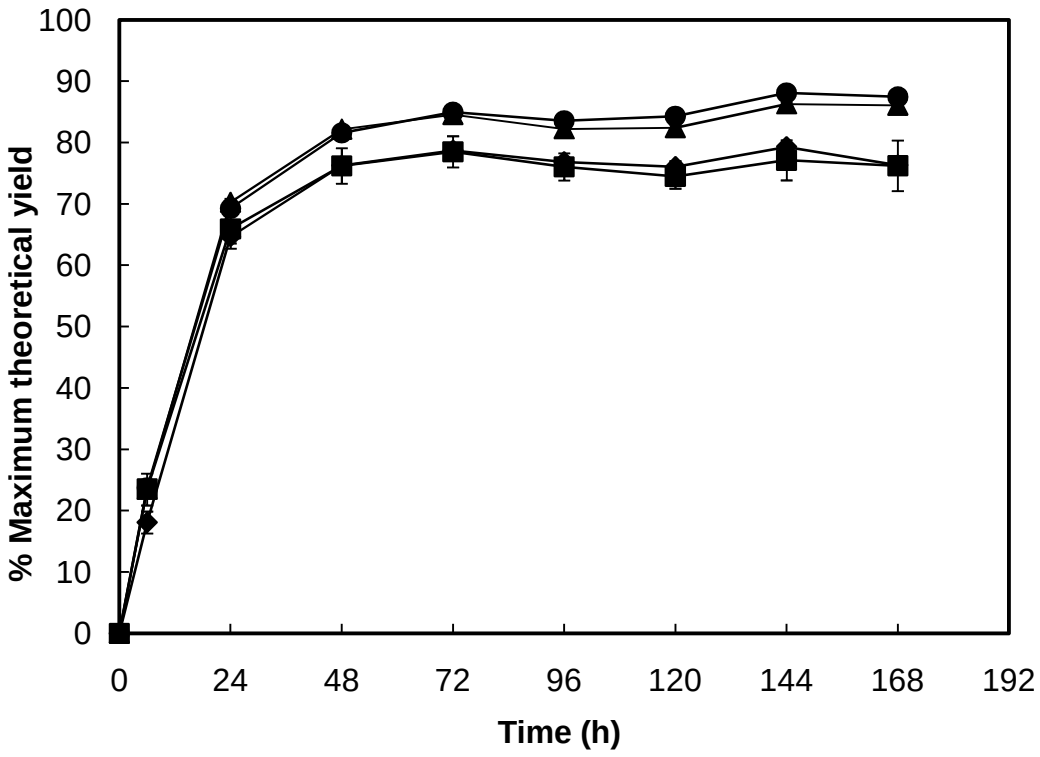

Fig. 5.5 Percentage of maximum ethanol theoretical yield using *K. marxianus* IMB3 with 8% pretreated switchgrass and Accellerase 1500 at 0.7 mL g^{-1} glucan at different temperatures: (♦) 37°C, (■) 41°C, (▲) 45°C, (●) 37°C with *S. cerevisia*e D5A (n=3).

In the present study, acetic acid profiles during SSFs with IMB3 and D_5A at various temperatures are shown in Fig. 5.6. The final acetic acid concentrations in the medium with IMB3 at 37, 41 and 45°C were 5.7, 6.3 and 3.6 g L^{-1}, respectively. Only about 0.8 g L^{-1} of acetic acid was produced with *S. cerevisiae* D_5A, which was lower than SSF with IMB3 ($p < 0.05$). The acetic acid produced in SSFs with IMB3 after 120 h and

41°C was significantly higher than at 37°C and 45°C ($p < 0.05$).

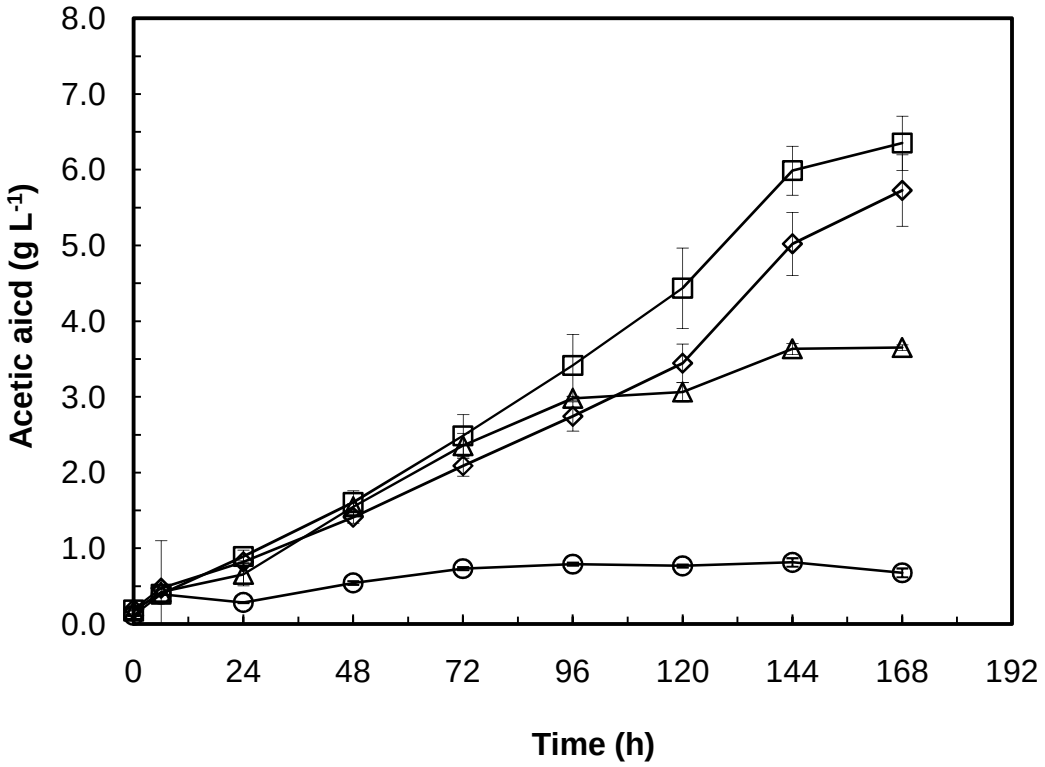

Fig. 5.6 Acetic acid profiles using *K. marxianus* IMB3 and 8% pretreated switchgrass and Accellerase 1500 at 0.7 mL g^{-1} glucan at different temperatures: (◊) 37°C, (□) 41°C, (Δ) 45°C, (○) 37°C with *S. cerevisia*e D5A (n=3).

Acetic acid can form during fermentation of sugars by yeast and inhibit their growth. Narendranath et al.(2001) reported that *S. cerevisiae* was not able to grow with 6 g L^{-1} acetic acid. Acetic acid can also be formed during the enzymatic hydrolysis of hemicelluloses (Öhgren et al., 2007; Sun & Cheng, 2002). It was found that acetic acid concentrations obtained after 168 h of hydrolysis of pretreated switchgrass with Accellerase 1500 were lower than 0.5 g L^{-1} (Appendix A). This showed that the increase in acetic acid concentration during SSFs was mostly due to IMB3 metabolism and not from the enzymatic hydrolysis of hemicelluloses. The reason for IMB3 utilizing glucose

after 72 h for production of acetic acid instead of ethanol is unknown.

Xylitol was also formed during SSFs with IMB3. At the end of SSFs, 1.3 g L^{-1} of xylitol was measured at 37°C, which was 29% and 24% higher than at 41°C and 45°C, respectively. Less than 1 g L^{-1} of succinic acid was obtained with IMB3 in all SSFs at the three temperatures used. However, glycerol production increased with increasing the temperature with IMB3. The highest glycerol concentration of 3.2 g L^{-1} was obtained at 45°C compared to 2.4 g L^{-1} at 37°C.

5.4 Effect of increased solid loading and fed-batch strategy on SSFs

In order to produce high ethanol concentrations, SSFs were performed at 12% solid loading in a batch and fed-batch processes. Four different feeding strategies labeled A through D were evaluated in this experiment as described in the Materials and Methods section. With strategy A, saccharification occurred at a higher rate compared to the other three strategies, which is evident from the residual glucose at 6 h (Fig. 5.7). About 11.0 to 19.6 g L^{-1} of glucose was noticed at 6 h in all SSFs. SSFs labeled A and B has improved hydrolysis of glucan compared to strategies C and D, which resulted in more glucose accumulation at 6 h. The highest glucose (19.6 g L^{-1}) was accumulated in strategy A in which 4.8 mL of enzyme was added. With same amount of enzyme added in SSF strategy B, 16.5 g L^{-1} of glucose was accumulated after 6 h. Glucose accumulation in SSFs with 12% solids started after 48 h (i.e., about 24 to 48 h earlier than SSFs with 8% solids) as shown in Figs. 5.1, 5.4 and 5.7. This was due to the high solid loading used.

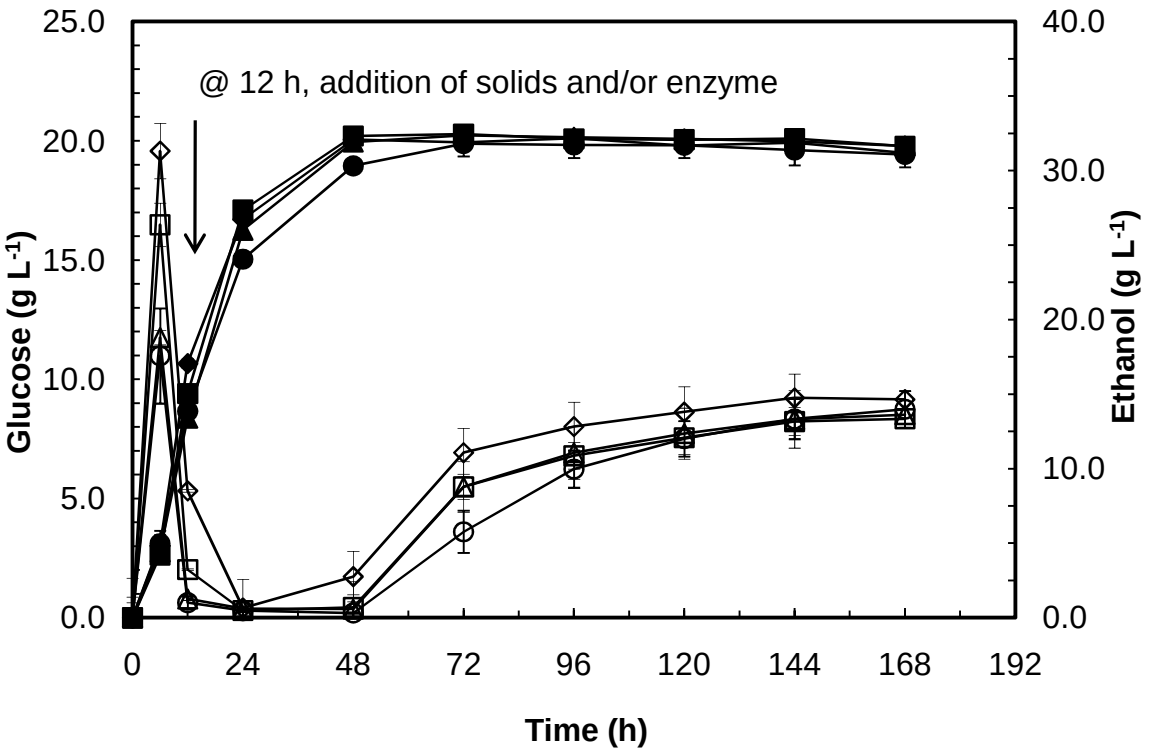

Fig. 5.7 Glucose (open symbols) and ethanol (solid symbols) profiles using *K. marxianus* IMB3 with SSFS at 45°C and various feeding strategies: (◊) A-batch mode (12% solids and 4.85 mL enzyme), (□) B-fed-batch mode (8% solids and 4.85 mL enzyme at 0 h plus 4% solids at 12 h), (Δ) C-fed-batch mode (8% solids and 3.23 mL enzyme at 0 h plus 4% solids and 1.62 mL enzyme at 12 h), (○) D-fed-batch mode (8% solids and 3.23 mL enzyme at 0 h plus 4% solids at 12 h) (n=3).

Ethanol concentrations in SSFs with the four feeding strategies were within 15% of each other at 6 h. Ethanol concentration of 17.1 g L^{-1} was obtained at 12 h with 12% solids in SSF batch mode (strategy A), which was 13%, 27% and 23% more than feeding strategies B, C and D, respectively (Fig. 5.7). Also, SSFs for strategies C and D were started with 8% solids and 0.7 mL g^{-1} glucan and resulted in similar concentrations of glucose and ethanol at 12 h. After 12 h, only 4% solids were added to the flasks with SSFs strategies B and D, while 4% solids with enzyme loading of 0.7 mL g^{-1}glucan were

added in the flasks with SSF strategy C. The addition of 4% solids in SSFs with strategy B produced the highest amount of ethanol (27.3 g L^{-1}) at 24 h ($p < 0.05$). The addition of 4.85 mL of enzyme at 0 h in strategy B resulted in high saccharification rate and better mixing in fed-batch mode that could have facilitated more ethanol production in the first 48 h compared to other strategies. In SSFs with strategy D, 24.1 g L^{-1} ethanol was obtained at 24 h, which was the lowest of the strategies ($p < 0.05$). Ethanol concentrations between 30 g L^{-1} and 32 g L^{-1} (Fig. 5.7), corresponding to ethanol yields between 77% and 81% MTY, were obtained at 48 h in all feeding strategies (Fig. 5.8).

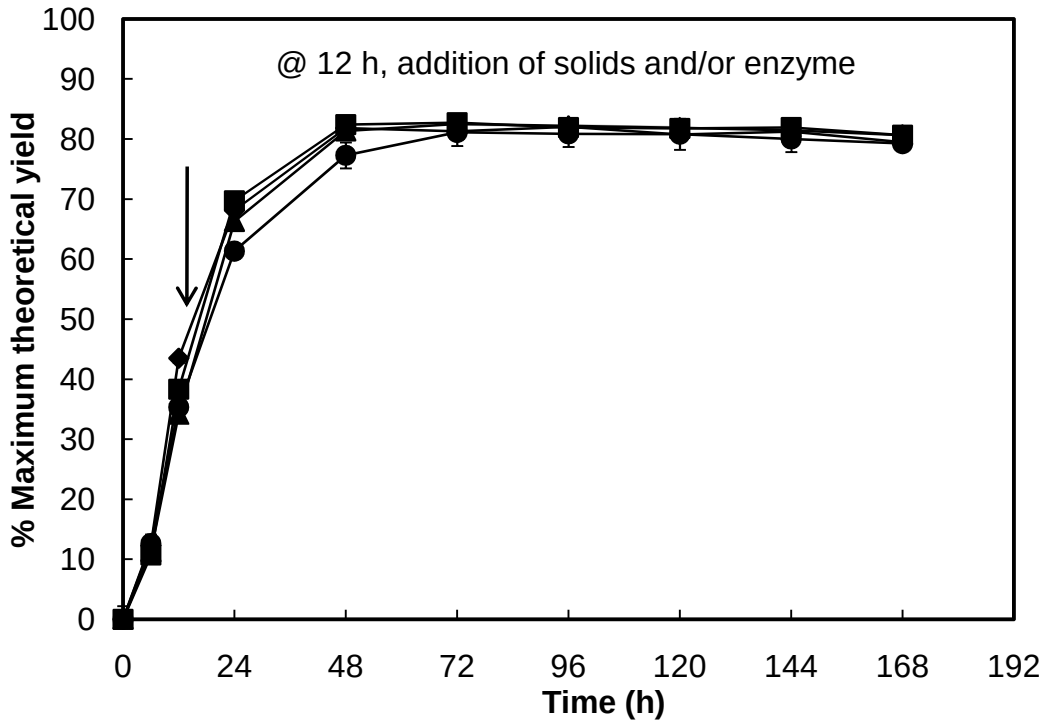

Fig. 5.8 Percentage of maximum ethanol theoretical yield using *K. marxianus* IMB3 with SSFS at 45°C and various feeding strategies: (♦) A-batch mode (12% solids and 4.85 mL enzyme), (■) B-fed-batch mode (8% solids and 4.85 mL enzyme at 0 h plus 4% solids at 12 h), (▲) C-fed-batch mode (8% solids and 3.23 mL enzyme at 0 h plus 4% solids and 1.62 mL enzyme at 12 h), (●) D-fed-batch mode (8% solids and 3.23 mL enzyme at 0 h plus 4% solids at 12 h) (n=3).

There were no significant differences in the amounts of ethanol produced by the four feeding strategies after 72 h ($p < 0.05$). About 32 g L^{-1} ethanol was produced in all strategies after 72 h, which was over 80% MTY (Fig. 5.8). Of particular significance were the results of SSFs with feeding strategy D in which lower total enzyme loading was used, but similar ethanol concentrations were obtained compared to other feeding strategies with higher enzyme loadings.

The findings in the current study were similar to those obtained by Hoyer et al. (2010), who used steam pretreated softwoods and a similar enzyme feed strategy in SSFs at 10% (w/v) and 14% (w/v) solids. Hoyer et al. (2010) found that with 14% (w/v) solids, ethanol yield increased from 51% in batch mode to 61% in fed-batch mode (enzyme added initially and solids added at various times), and to 58% in a second fed-batch mode (solids and enzymes were mixed and added at various times).

Acetic acid concentrations in all SSFs were lower than 2.5 g L^{-1}after 168 h (Fig. 5.9). The highest acetic acid concentration of 2.3 g $L^{-1}$was obtained with feeding strategy D, which was significantly different from other strategies ($p < 0.05$) and the lowest concentration of 1.7 g L^{-1} was obtained with feeding strategy A, which was significantly lower than for other feeding strategies after 72 h of SSF ($p < 0.05$). However, there were insignificant differences in the acetic acid concentrations with feeding strategies B and C ($p > 0.05$). Acetic acid concentrations obtained in batch mode with 12% solids (Fig. 5.9) were over twofold lower than in SSFs with 8% solids (Fig. 5.3).

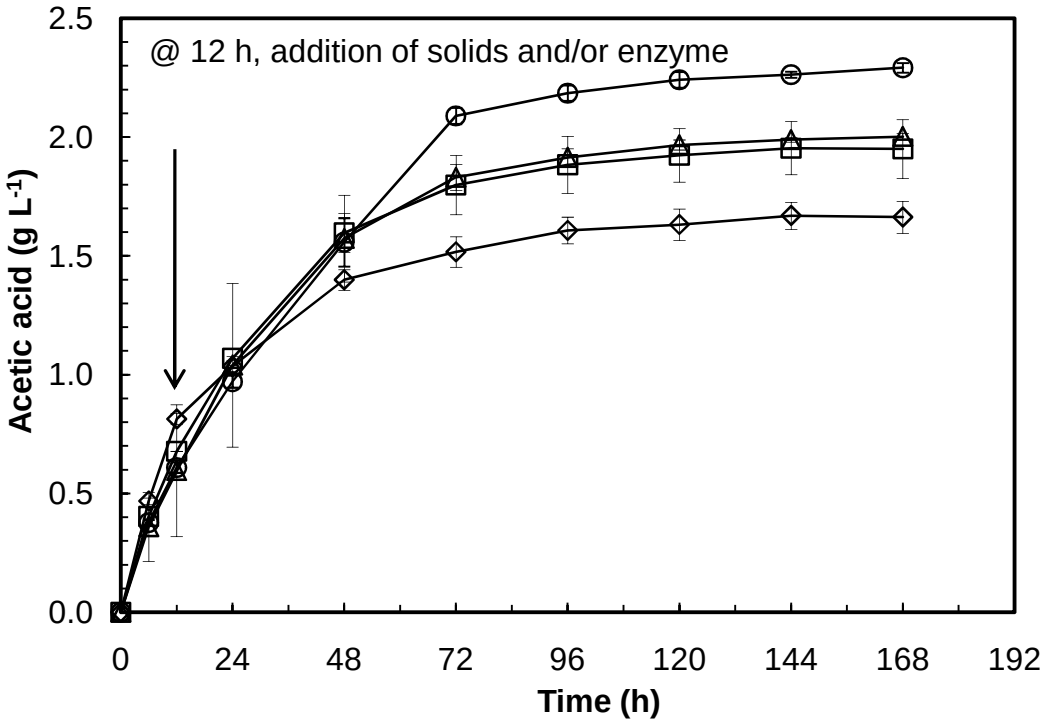

Fig. 5.9 Acetic acid profiles using *K. marxianus* IMB3 with SSFs at 45°C and various feeding strategies: (◊) A-batch mode (12% solids and 4.85 mL enzyme), (□) B-fed-batch mode (8% solids and 4.85 mL enzyme at 0 h plus 4% solids at 12 h), (Δ) C-fed-batch mode (8% solids and 3.23 mL enzyme at 0 h plus 4% solids and 1.62 mL enzyme at 12 h), (○) D-fed-batch mode (8% solids and 3.23 mL enzyme at 0 h plus 4% solids at 12 h) (n=3).

5.5 Mass balance calculations

Mass balance calculations were performed on glucose, xylose and lignin to ensure that all end-products were accounted for in the pretreatment process and SSFs. A sample calculation of the mass balance determination is shown in Appendix B. The water loss in hydrothermolysis pretreatment of switchgrass was less than 1%. The hydrothermolysis pretreatment altered the glucan and xylan composition of the switchgrass (Table 5.1). The glucose and xylose balances for the pretreatment process closed at 109.5% and 103.7%,

respectively. This means that 10% and 4% over estimations of glucose and xylose, respectively, resulted from the pretreatment process. This is could be due to experimental errors associated with the analysis of sugar using HPLC and the determination of the composition of switchgrass before and after pretreatment.

The conversion efficiency of glucan and mass balances on lignin in all SSFs experiments are shown in Table 5.2. The conversion efficiency of glucan during all SSFs was higher than 95%. Mass balances on lignin closed to 100 ± 10% in all SSFs experiments. Glucose balances for the effect of enzyme loading experiment close to 92.8%, 99.7% and 110.4% with enzyme loadings of 0.3, 0.5 and 0.7 mL g^{-1} glucan, respectively. Xylose balances closed to 56.5%, 73.2% and 82.4% with enzyme loadings of 0.3, 0.5 and 0.7 mL g^{-1} glucan, respectively. The reason for not closing the xylose balance within 100 ± 10% could be due to xylan hydrolysis to oligomers that were not detected by the HPLC method used. It is unlikely that xylose was utilized for metabolism by IMB3. The analysis of residual pretreated solids after all SSFs showed that there was no xylan present in the solids which indicate a complete hydrolysis of xylan.

The glucose mass balances closed to 103.6%, 105.4% and 108.8% for SSFs at 37, 41 and 45°C, respectively. However, xylose mass balances closed to 63.6%, 55.0% and 72.8% for SSFs at 37, 41 and 45°C, respectively. For the effect of solid loading and feeding strategy experiment, glucose mass balances closed to 104.6%, 103.4%, 103.7% and 102.7% with strategies A, B, C and D, respectively. However, xylose balances closed to 44.8%, 47.6%, 44.8%and 40.6% for SSFs with strategies A, B, C and D, respectively.

Table 5.2 Glucan conversion efficiency and lignin balance for various SSF experiments using *K. marxianus* IMB3.

	Initial glucan ($g\ L^{-1}$)	Initial lignin ($g\ L^{-1}$)	Final glucan ($g\ L^{-1}$)	Final lignin ($g\ L^{-1}$)	Glucan conversion (%)	Lignin balance closed (%)
Effect of enzyme loading						
Enzyme loading ($mL\ g^{-1}$ glucan)[a]						
0.3	46.4	28.0	2.1	25.3	95.4	91.2
0.5	46.4	28.0	1.4	27.5	97.1	98.1
0.7	46.4	28.0	1.2	27.9	97.4	99.7
Effect of temperature[b]						
Temperature (°C)						
37	46.4	28.0	1.5	28.8	96.7	103.0
41	46.4	28.0	1.2	28.2	97.4	100.7
45	46.4	28.0	1.0	29.2	97.8	104.3
Effect of solid and enzyme feeding strategy						
Strategies[c]						
A	69.6	42.0	1.2	42.6	98.3	101.4
B	69.6	42.0	1.9	39.7	97.3	94.5
C	69.6	42.0	2.2	38.0	96.9	90.6
D	69.6	42.0	1.7	43.2	97.6	102.8

[a] Solid loading of 8% and Accellerase 1500 (enzyme activity = 82.2 FPU mL^{-1} enzyme)

[b] Solid and enzyme loadings were 8% and 0.7 mL g^{-1} glucan

[c] Strategy A: batch mode with 12% solids and 0.7 mL g^{-1} glucan added at t = 0 h; B: fed-batch mode with 8% solids and 0.7 mL g^{-1} glucan based on 12% solids added at t= 0 h plus 4% solids added at t = 12 h; C: fed-batch mode with 8% solids and 0.7 mL g^{-1} glucan added at t= 0 h plus 4% solids and 0.7 mL g^{-1} glucan added at t = 12 h; D: fed-batch mode with 8% solids and 0.7 mL g^{-1} glucan added at t= 0 h plus 4% solids and no additional enzyme added at t = 12 h

CHAPTER 6

CONCLUSIONS

- Hydrothermolysis pretreatment resulted in pretreated switchgrass solids that contained 57.7% glucan, 5.0% xylan and 35.1% lignin. About 15.8% glucan and 87.7% xylan from the switchgrass solids were removed into the prehydrolyzate after pretreatment. The water loss in hydrothermolysis pretreatment of switchgrass was less than 1%. The glucose and xylose balances for the pretreatment process closed at 109.5% and 103.7%, respectively.
- The thermotolerant strain *K. marxianus* IMB3 produced significantly more ethanol in SSFs with 8% solids as the enzyme Accellerase 1500 (Genencor, NY, USA) loading increased from 0.3 to 0.7 mL g^{-1} glucan ($p < 0.05$). The enzyme loading of 0.7 mL g^{-1} glucan resulted in the highest ethanol concentration of 22.3 g L^{-1}, which was equivalent to 85% maximum theoretical yield (MTY). About 2.3 g L^{-1} glucose was accumulated and 4 g L^{-1} acetic acid was produced after 168 h of SSF. In addition, small amounts of xylitol, glycerol and succinic acid were formed during SSFs with IMB3.

- *K. marxianus* IMB3 produced more ethanol during SSF of 8% solids at the optimum enzyme loading of 0.7 mL g^{-1} glucan as the temperature was increased from 37°C to 45°C. The highest ethanol concentration of 23.0 g L^{-1} (86.3% MTY) was obtained in SSF at 45°C compared to about 20 g L^{-1} ethanol (77% MTY) at 37°C and 41°C. Negligible amounts of glucose accumulated in SSF with IMB3 at 37°C and 41°C compared to about 1.4 g L^{-1} glucose accumulated at 45°C. Ethanol production by the thermophilic yeast *K. marxianus* IMB3 in SSF at 45°C was similar to *S. cerevisiae* D_5A that cannot grow above 37°C. Acetic acid production by *K. marxianus* IMB3 in SSFs increased when the temperature was increased from 37°C to 41°C. However, acetic acid production by IMB3 at 45°C was about 50% lower than at 41°C.

- No significant differences in the amount of ethanol produced were observed in SSFs operated in batch or fed-batch modes at 45°C and 12% solids using IMB3 ($p > 0.05$). About 32 g L^{-1} ethanol (81% MTY) was produced in SSFs with IMB3 using a total solid loading of 12% in all batch and fed-batch feeding strategies. About 9 g L^{-1} glucose and between 1.7 and 2.5 g L^{-1} acetic acid accumulated at the end of SSF with all feeding strategies. Moreover, results also showed that using fed-batch mode with 12% solids, the enzyme loading was decreased by 33% of the optimum loading for batch SSF.
- Over 90% of the ethanol produced by *K. marxianus* IMB3 occurred during the first 48 to 72 h in all SSFs experiments. IMB3 fermentation ability at 45°C stopped after about 96 h, which could be due to multiple stresses on IMB3 cells such as high concentrations of ethanol and acetic acid in the medium.

- Mass balances on SSFs were done to ensure that all end-products were accounted for in the SSF. The conversion efficiency of glucan during all SSF experiments was higher than 95%. Also, lignin and glucose balances closed to 100 ± 10% in all SSFs experiments.
- Xylose balances for effect of enzyme loading, temperature and feeding strategies experiments closed from 57% to 82% and from 55% to 83% and from 41% to 48%, respectively. The reason for xylose balance not closing to 100% could be due to xylan hydrolysis to oligomers that were not detected using the HPLC method used.

CHAPTER 7

FUTURE WORK

Several areas could be investigated to improve ethanol yields and thermotolerance of IMB3. Inoculation could be done at 12 h or 24 h instead of at time zero. This will allow the enzymatic hydrolysis of glucan to make glucose more readily available for IMB3 at the time of inoculation. This could also prolong the thermotolerance of IMB3 and increase ethanol yields. From the previous studies, it was evident that IMB3 was capable of SSF at 12% solid loading. The solid loadings could be further increased to 16% or 20%. Furthermore, solids can be added in fed-batch strategy to facilitate mixing and prevent the substrate inhibition of the enzyme.

SSFs could be further improved by addition of nutrients and cells during the course of fermentation. Another study that can be performed is the SSCF of pretreated switchgrass slurry. IMB3 could be adapted to the inhibitors present in the prehydrolyzate and can be used in SSCF process. Microorganisms such as *Zymomonas mobilis* and *Escherichia coli* KO11, which are capable of utilizing C5 sugars can be further added to metabolize xylose present in the medium. This could greatly improve the ethanol concentrations and allows complete utilization of C5 and C6 sugars in the medium, thereby increasing ethanol yields.

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APPENDIX A

A.1 Enzymatic hydrolysis of switchgrass at various temperatures

For effect of temperature on enzymatic hydrolysis of switchgrass by Accellerase 1500, 8% solids were added into the flasks. Then, 5 mL citrate buffer (pH 5.5), 20 mL of 50 X concentrated YFM, 50 mg L^{-1} chloramphenicol were added. The enzyme loading was 0.7 mL g^{-1} glucan. The flasks were incubated in an orbital shaker at 37, 41 and 45°C. Sampling and analysis were performed as described in Materials and Method section.

Glucan hydrolysis rate increased with the increase in temperature from 37 to 45°C (Fig. A.1). At 45°C, about 54% of glucan was converted to glucose within 24 h which was 4% and 13% higher than the glucan conversion at to 41 and 37°C, respectively. The hydrolysis rate was high at 6 h at the three temperatures used. Then, the hydrolysis rate decreased, which was due to the accumulation of glucose released during the hydrolysis of glucan. Unlike SSFs, glucose was not removed during the hydrolysis of glucan, which resulted in reduction in the enzyme activity. At the end of the hydrolysis run at 45°C, 95% of glucan was hydrolyzed to glucose, which was 7% and 14% higher than at 41 and 37°C, respectively. With hydrolysis at the three temperatures used, the maximum acetic acid concentration was 0.5 g L^{-1}, which was obtained after 144 h. This means that acetic acid production during SSF was due to IMB3 metabolism of glucose released from glucan and not due to hydrolysis of glucan.

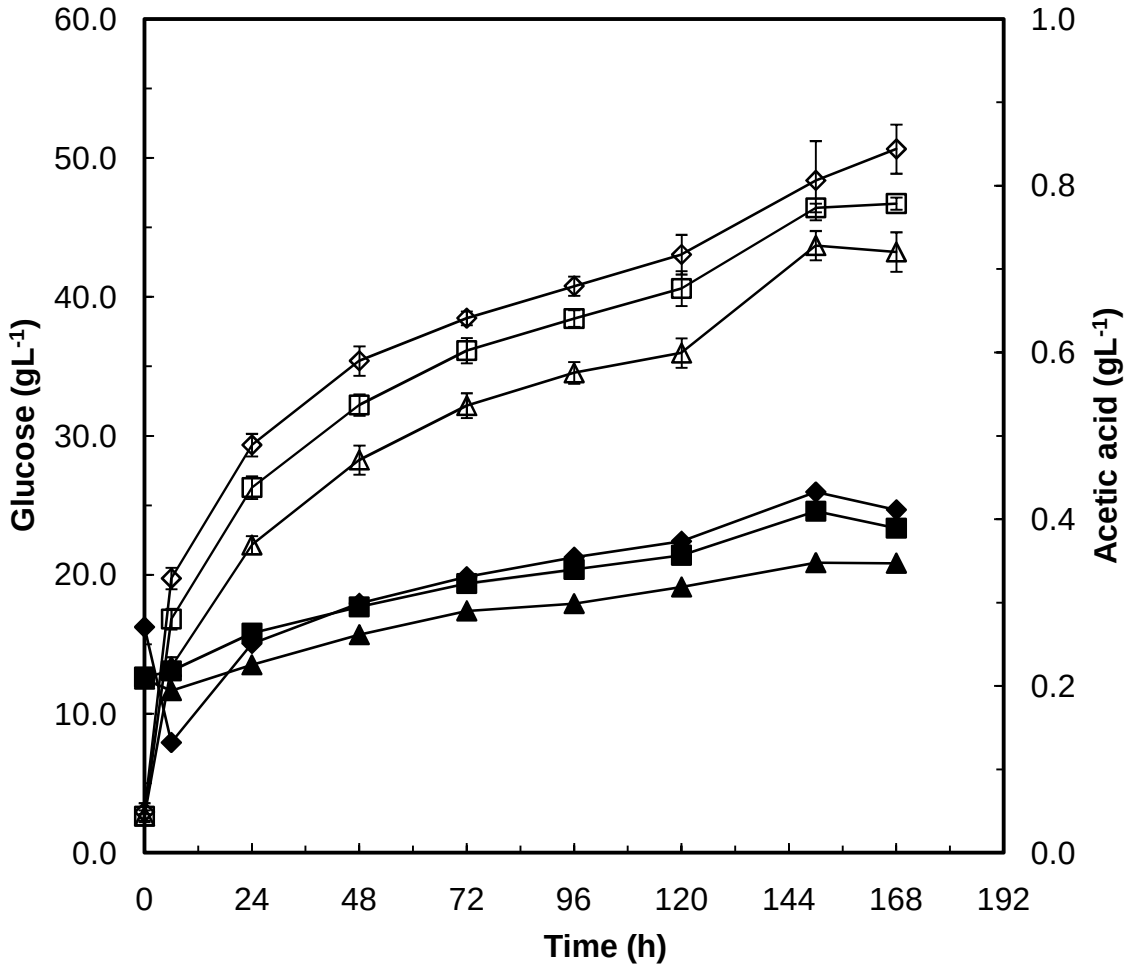

Fig. A.1 Glucose (open symbols) and acetic acid (solid symbols) profiles during hydrolysis of 8% pretreated switchgrass using Accellerase 1500 (0.7 mL g^{-1} glucan) at various temperatures: (Δ) 37°C, (□) 41°C, (◊) 45°C (Note: 1 mL of enzyme = 82.2 FPU).

A.2 Effect of re-inoculation and increased media supplementation on SSF

In order to determine the effect of addition of media components and re-inoculation, an SSF was performed the same way as described in the Materials and Methods section but using 20 mL of 50X YFM. Substrate and enzyme loadings were 8% and 0.7 mL g^{-1} glucan in all flasks. The temperature was maintained at 45°C and agitation

speed at 130 rpm. The SSFs were performed in four sets, with each set maintained in triplicate. All SSFs started with similar medium and initial cell concentrations. After 48 h of the SSF, medium and/or cells were added as follows: first set of flasks were labeled as (1XC, 1XM), in which 1X medium (20 mL of 50X YFM) and 1X cells were added. Second set of flasks were labeled as (1XC, 0.5XM), in which 0.5X (10 mL of 50X YFM) medium and 1X cells were added. Third set of flasks were labeled (1XC), in which only 1X cells was added. Fourth set of flasks were labeled (1XM), in which only 1X medium (20 mL 50X YFM) was added. The pH of the flasks was measured before addition of new medium or cells and then adjusted to 5.2 using 2N KOH.

Previous SSFs with concentrated medium (20 mL of 50X YFM) and 8% solids at 45°C resulted in accumulation of 9 g L^{-1} glucose at the end of SSF, which decreased the ethanol yield to 75%. This led to a hypothesis that the glucose accumulation was due to the lack of viable cells and/or depletion of nutrients at 45°C. In order to decrease the accumulation of glucose in the medium and improve ethanol yield at 45°C, SSFs were supplemented with fresh 50X YFM medium and/or new IMB3 cells after 48 h of SSF. Although all strategies used decreased the accumulation of glucose in SSFs to below 4 g L^{-1}, the (1XC, 1XM) and (1XM) additions reduced glucose to lowest level of 2.6 g L^{-1} (Fig. A.2).

The SSF inoculated with only cells (1XC) resulted in the highest ethanol concentration after 168 h (Fig. A.3).This was the only treatment that significantly affected the ethanol yields ($p < 0.05$). No significant changes in ethanol yields were noticed with the other three strategies from 72 h to 168 h as seen in Fig.A.4 ($p > 0.05$). It was found that fermentations using thermotolerant *K. marxianus* EMS-26 with nutrient

supplementation had no significant effect on ethanol yields (Ballesteros et al., 1994) . Suryawati et al. (2008) used hydrothermolysis pretreated switchgrass in an SSF at various temperatures using IMB4. The nutrient concentration was tripled to enhance the growth of IMB4 beyond 96 h. However, SSFs with increased nutrient concentration had decreased ethanol yield.

About 3.8 g L^{-1} of glucose was measured after 168 h with (1XC). The decrease in glucose concentrations in these flasks could be attributed to the dilution effect caused by the addition of media and/or cells. With the addition of 1XM, there was a 21% increase in volume of the fermentation broth, thereby diluting the enzyme. Since the proximity of enzyme-substrate is affected by the dilution of media, this could have been the reason for decreased hydrolysis rate in SSFs other than with 1XC. Also, glucose accumulation increased as the dilution decreased (Fig. A.2). This similar trend was also found for ethanol production. However, with SSFs that have relatively same volume such as (1XC, 1XM) and (1XM) had no significant difference on ethanol concentrations ($p < 0.05$) following the reinoculation and addition of media components. This proved that either addition of cells and media or only media to the flasks had no positive effect towards higher ethanol yields (Fig. A.4). Though flasks reinoculated with 1XC showed a decreased glucose accumulation compared to SSFs performed at 45°C with no addition of cells, the glucose was not necessarily used for product production. Ethanol yields obtained after 72 h with the four treatments were lower than with previous SSFs at 45°C without any addition of cells or nutrients at 48 h. About 1.7 g L^{-1} to 2.1 g L^{-1} acetic acid was produced in all flasks with the four strategies by the end of SSF (Fig. A. 5).

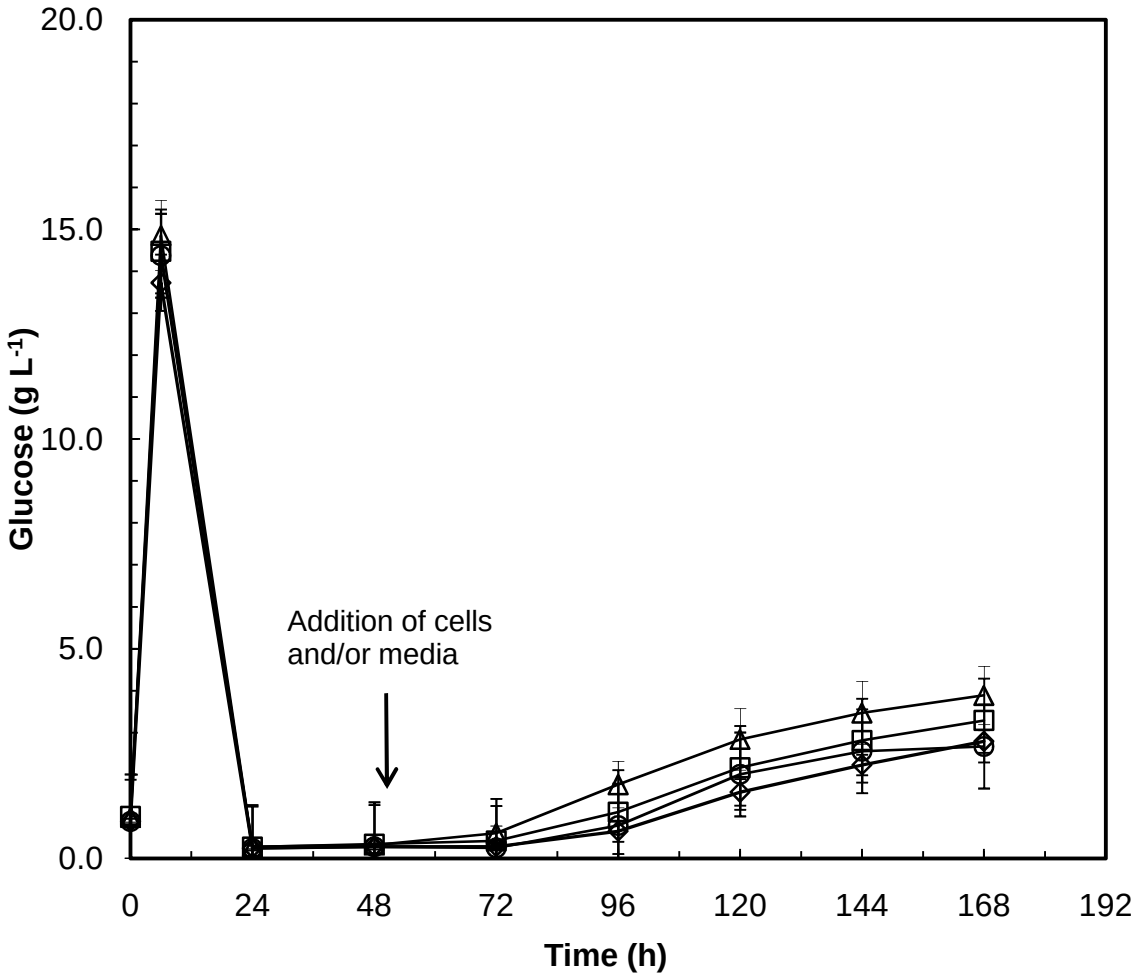

Fig. A.2 Glucose profiles obtained with SSFs at 45°C with different strategies using IMB3 with an enzyme loading of 0.7 mL g^{-1} glucan. (◊) 1XC, 1XM, (□)1XC, 0.5X M, (Δ) 1XC and (○) 1XM, (Note: data after 48 h were adjusted to take into consideration the effect of dilution caused by addition of medium (XM) and/or cells (XC), 1 mL of enzyme = 82.2 FPU).

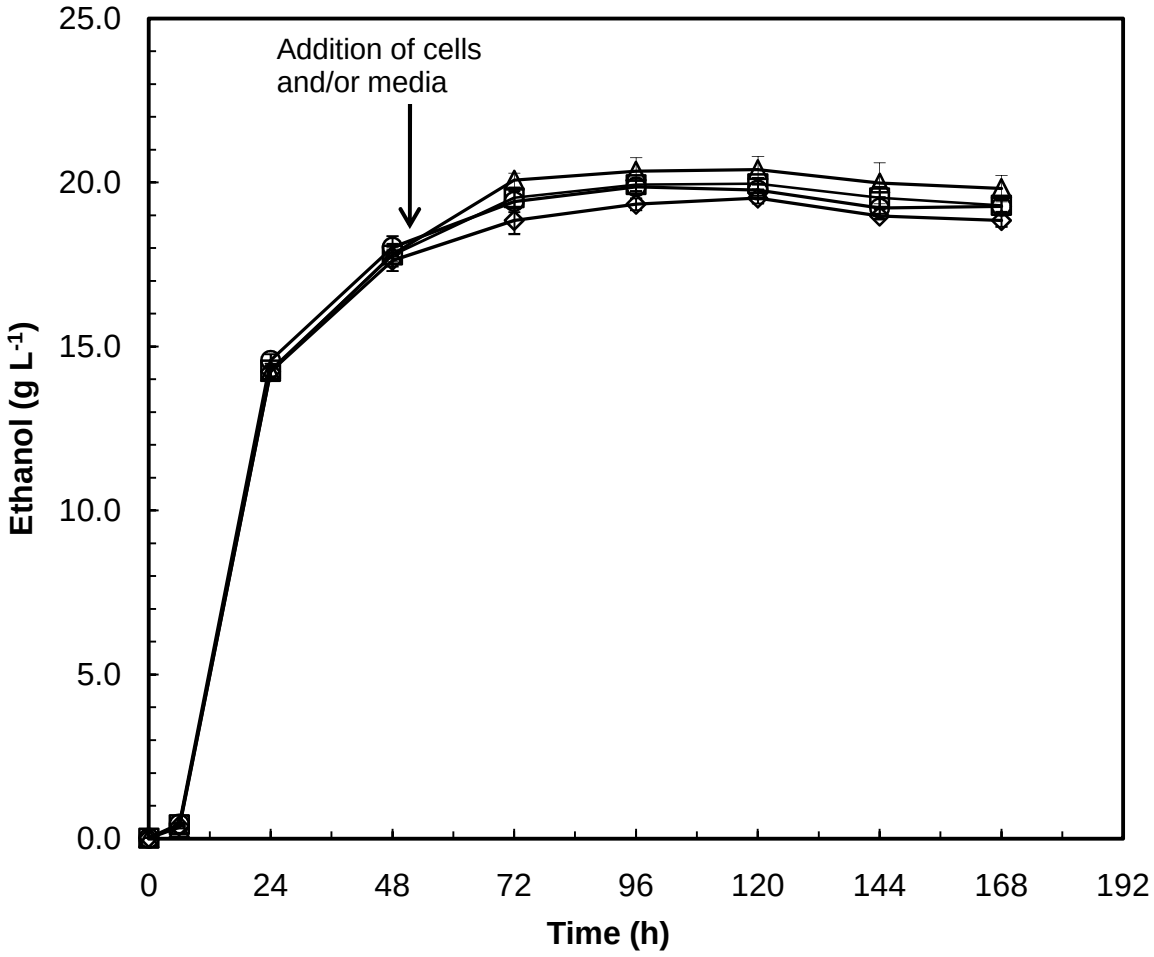

Fig. A.3 Ethanol profiles with SSFs at 45°C with different strategies using IMB3 with an enzyme loading of 0.7 mL g^{-1} glucan. (◊) 1XC, 1XM, (□) 1XC, 0.5X M, (Δ) 1XC and (○) 1XM, (Note: data after 48 h were adjusted to take into consideration the effect of dilution caused by addition of medium (XM) and/or cells (XC), 1 mL of enzyme = 82.2 FPU).

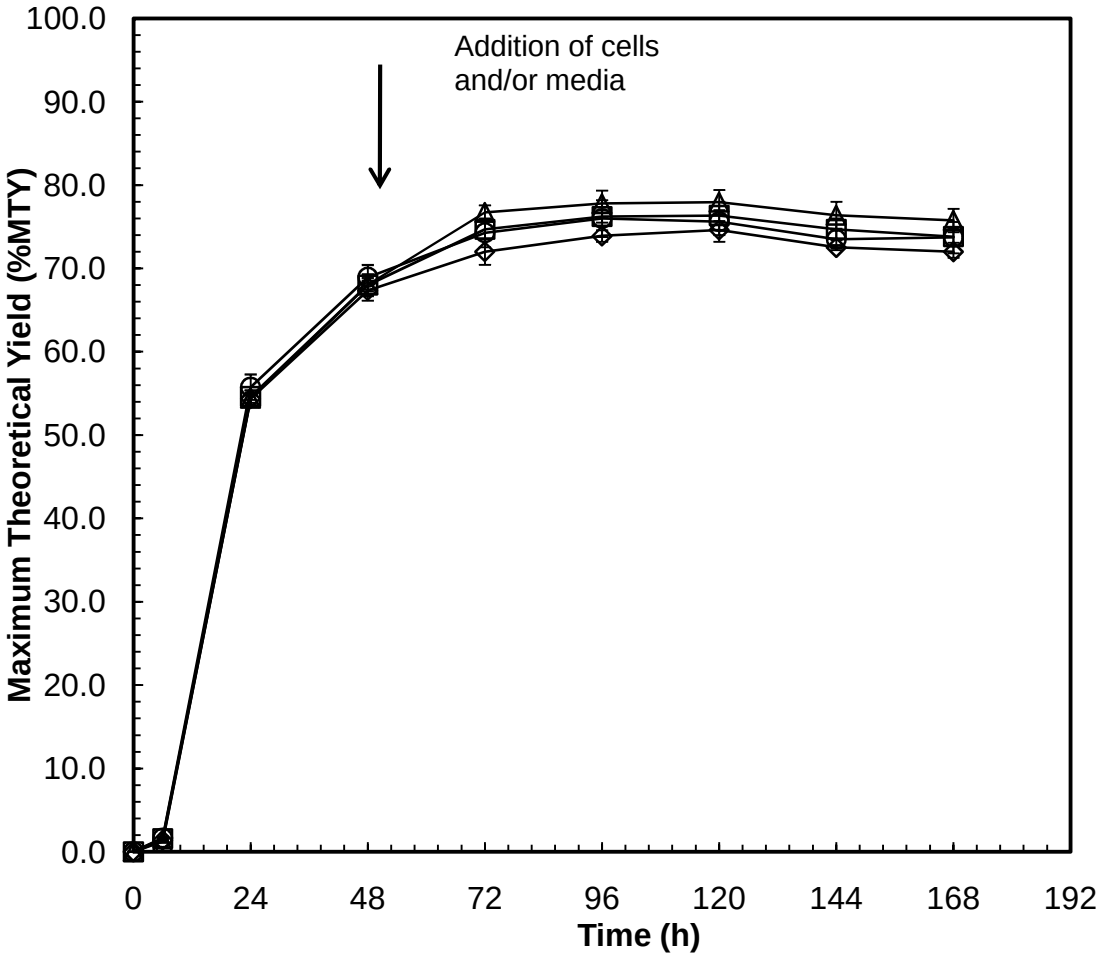

Fig.A.4 Maximum ethanol theoretical yield (% MTY) with SSFs at 45°C with different strategies using IMB3 with an enzyme loading of 0.7 mL g^{-1} glucan. (◊) 1XC, 1XM, (□) 1XC, 0.5X M, (Δ) 1XC and (○) 1XM (Note: data after 48 h were adjusted to take into consideration the effect of dilution caused by addition of medium (XM) and/or cells (XC), 1 mL of enzyme = 82.2 FPU).

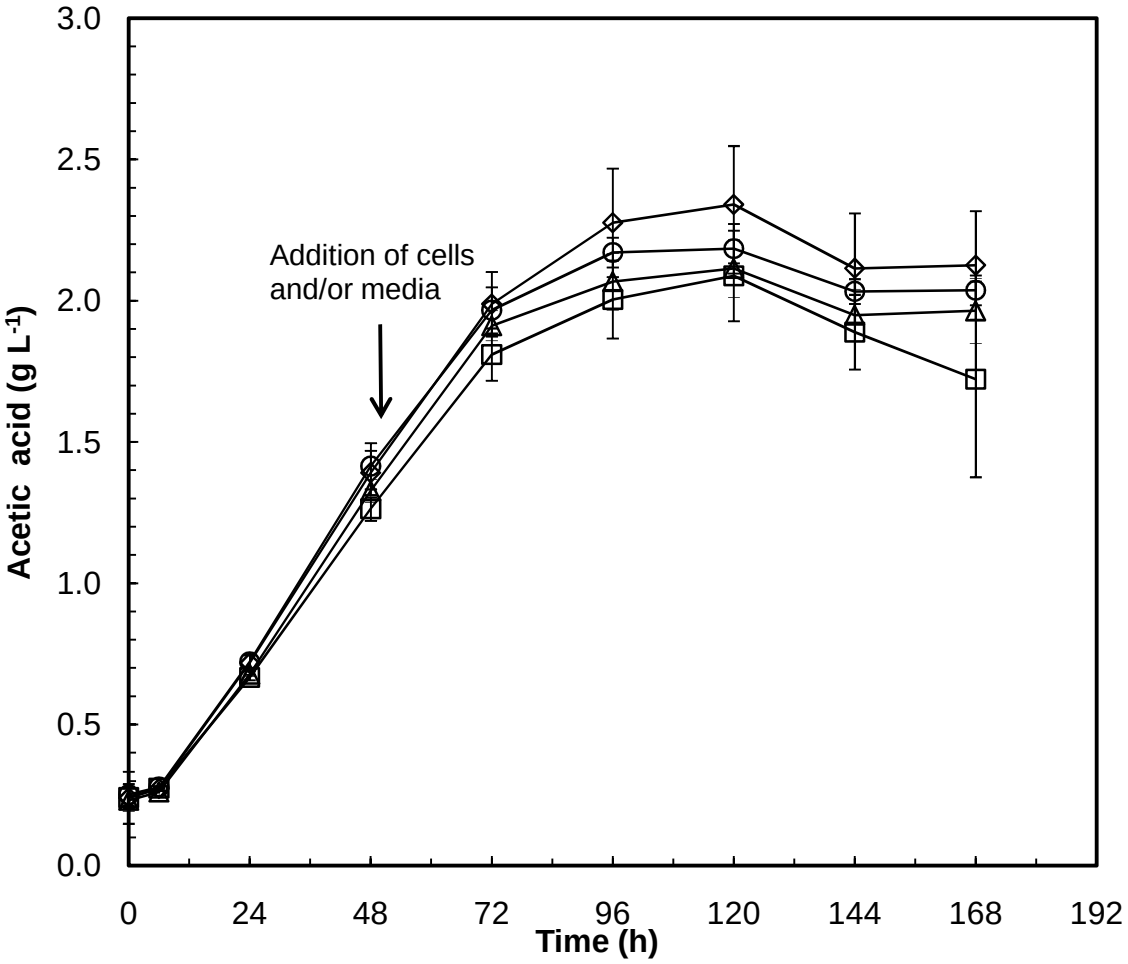

Fig. A.5 Acetic acid profiles with SSFs at 45°C with different strategies using IMB3 with an enzyme loading of 0.7 mL g^{-1} glucan. (◊) 1XC, 1XM,(□) 1XC, 0.5X M, (Δ) 1XC and (○) 1XM, (Note: data after 48 h were adjusted to take into consideration the effect of dilution caused by addition of medium (XM) and/or cells (XC), 1 mL of enzyme = 82.2 FPU).

A.3 Effect of enzyme loading on SSF using 10X media

The increase in Accellerase 1500 loading from 0.1 to 1.1 mL g^{-1} glucan showed an increase in glucan hydrolysis. Glucose concentrations measured after 6 h showed that there was an increased rate of glucan hydrolysis with increased enzyme loading (Fig.

A.6). Glucose accumulation was less than 0.5 g L^{-1} in SSFs with enzyme loadings of 0.1 to 0.5 mL g^{-1} glucan from 24 to 96 h. However, the glucose accumulation with enzyme loadings of 0.7 mL g^{-1} glucan and higher showed an increasing trend from 48 h until the end of SSFs. Residual glucose present in the flasks after 168 h was 0.2, 4.8, 4.5, 3.6, 5.16 and 5.2 g L^{-1} for enzyme loadings 0.1, 0.2, 0.3, 0.5, 0.7, 0.9, and 1.1 mL g^{-1} glucan, respectively. The maximum ethanol concentration obtained in enzyme controls was 0.05 g L^{-1}, which showed that the enzyme does not contain significant amount of residual glucose. Glucose accumulation at the end of SSF showed the inability of IMB3 to utilize all the glucose after 72 h.

A clear increasing trend has been noticed for ethanol within the range of enzyme loadings from 0.1 to 0.5 mL g^{-1} glucan. However, no significant differences in ethanol concentrations were observed after 72 h with enzyme loadings between 0.7 to 1.1 mL g^{-1} glucan ($p > 0.05$) as shown in Fig. A.7. The lowest ethanol concentration (5.2 g L^{-1}) was obtained with an enzyme loading of 0.1 mL g^{-1} glucan after 168 h which was equivalent to 20.2 % maximum theoretical yield (Fig. A.8). The highest ethanol concentration (22.4 g L^{-1}) was obtained with an enzyme loading of 0.7 mL g^{-1} glucan after 120 h which was equivalent to 87.4% maximum theoretical yield. Acetic acid production showed a mixed trend for enzyme loadings within the range 0.1 to 0.5 mL g^{-1} glucan (Fig. A.9). However, a decreasing trend was noticed within the range of enzyme loadings from 0.7 to 1.1 mL g^{-1} glucan. The final acetic acid concentrations were 2.9, 3.7, 3.4, 2.0, 1.8 and 1.7 g L^{-1} for enzyme loadings 0.1, 0.3, 0.5, 0.7, 0.9 and 1.1 mL g^{-1}glucan, respectively.

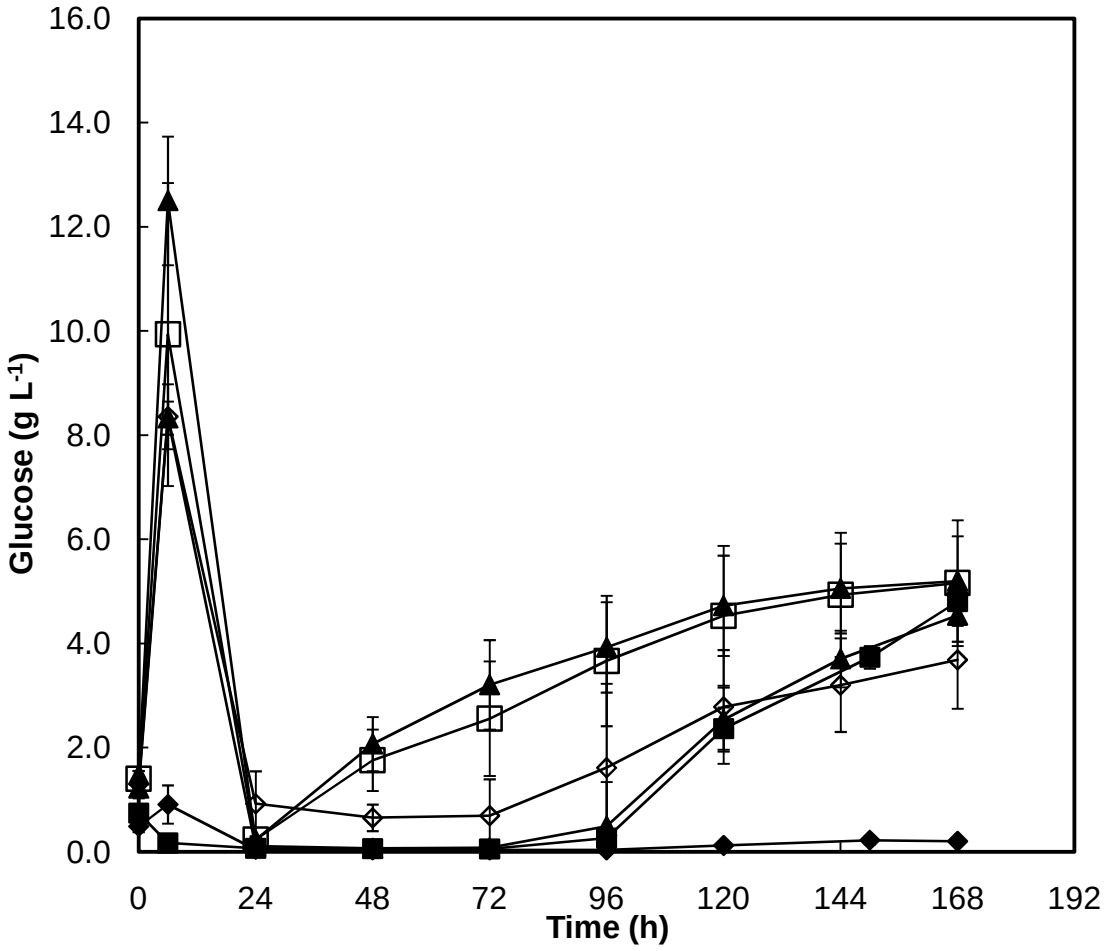

Fig A.6 Glucose profiles using *K. marxianus* IMB3 and 8% pretreated switchgrass in SSFs at 45°C and different enzyme loadings (mL g^{-1}glucan): (♦) 0.1, (■) 0.3, (▲) 0.5 ,(◊) 0.7, (□) 0.9, (Δ) 1.1 (n=3).

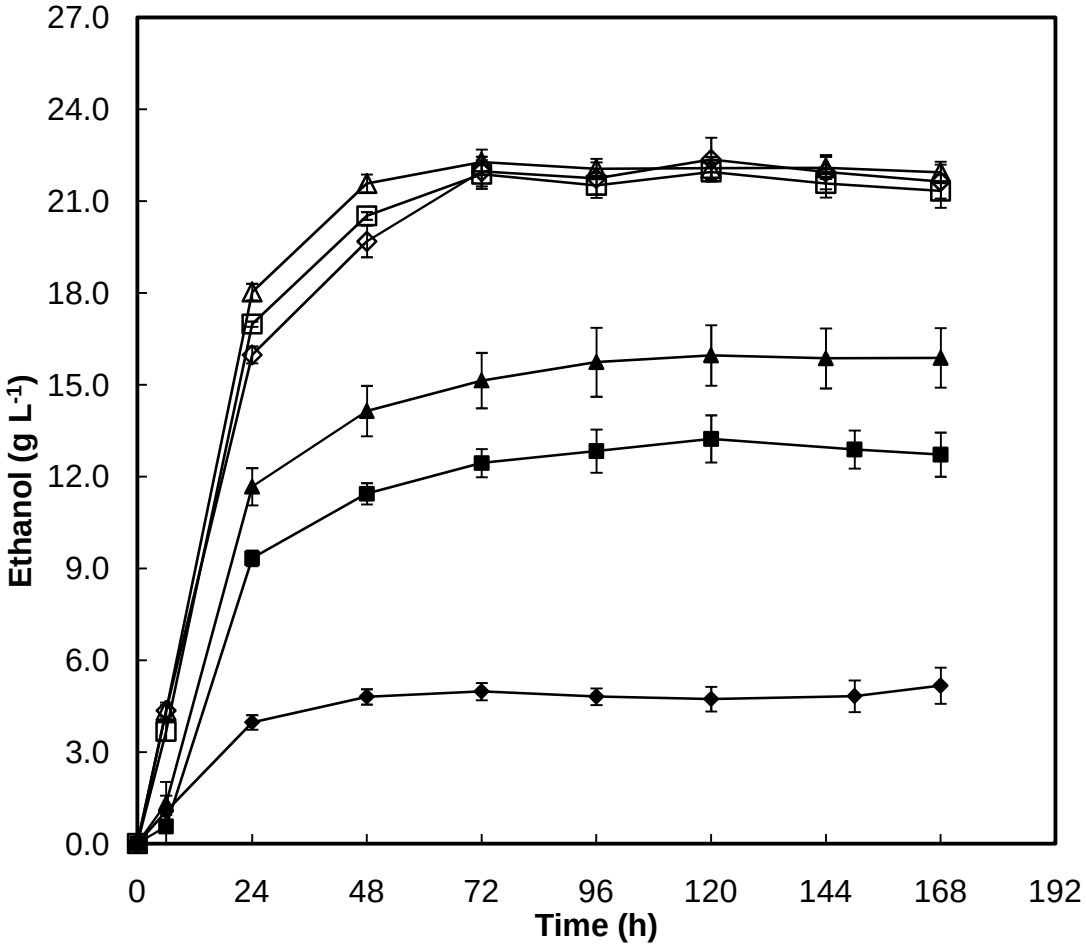

Fig A.7 Ethanol profiles using *K. marxianus* IMB3 and 8% pretreated switchgrass in SSFs at 45°C and different enzyme loadings (mL g^{-1}glucan): (♦) 0.1, (■) 0.3, (▲) 0.5 ,(◊) 0.7, (□) 0.9, (Δ) 1.1 (n=3).

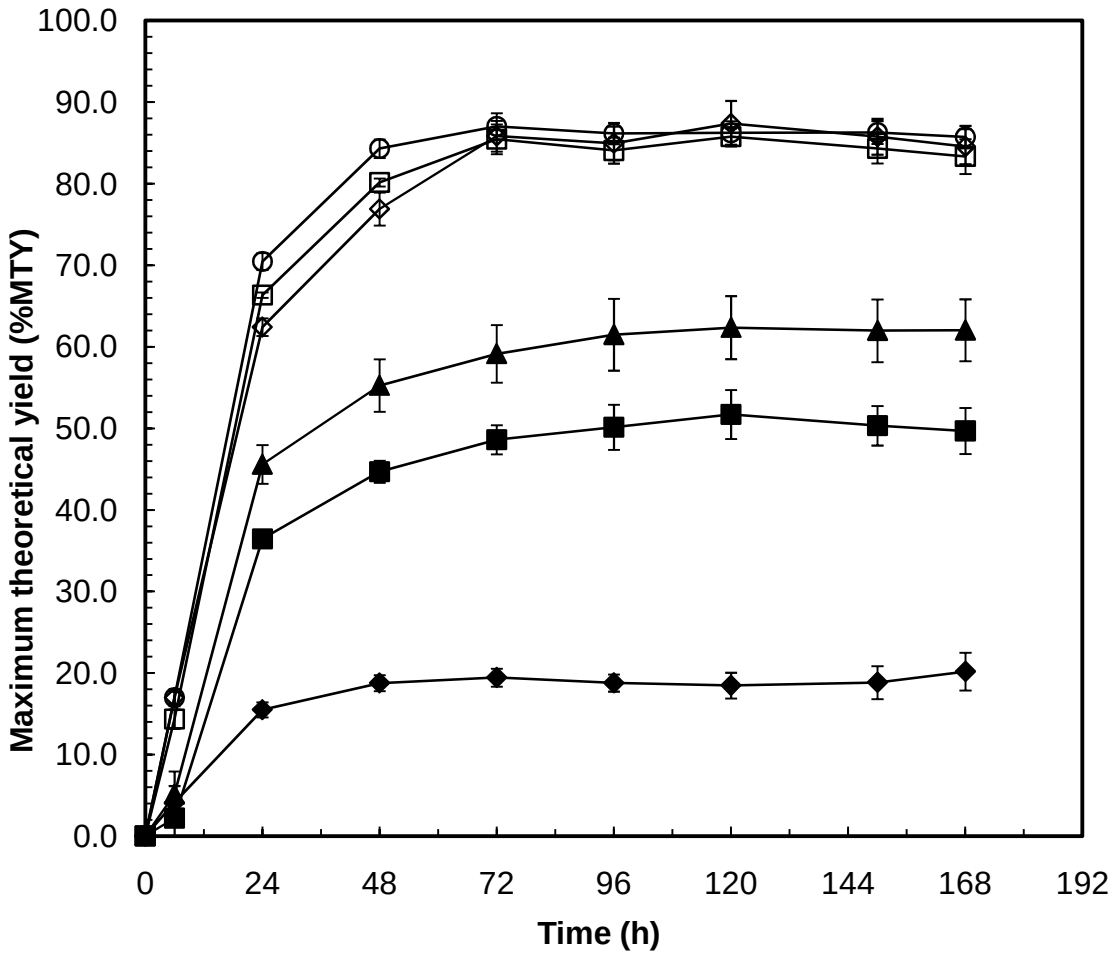

Fig A.8 Maximum theoretical yield profiles using *K. marxianus* IMB3 and 8% pretreated switchgrass in SSFs at 45°C and different enzyme loadings (mL g^{-1}glucan): (♦) 0.1, (■) 0.3, (▲) 0.5 ,(◊) 0.7, (□) 0.9, (Δ) 1.1 (n=3).

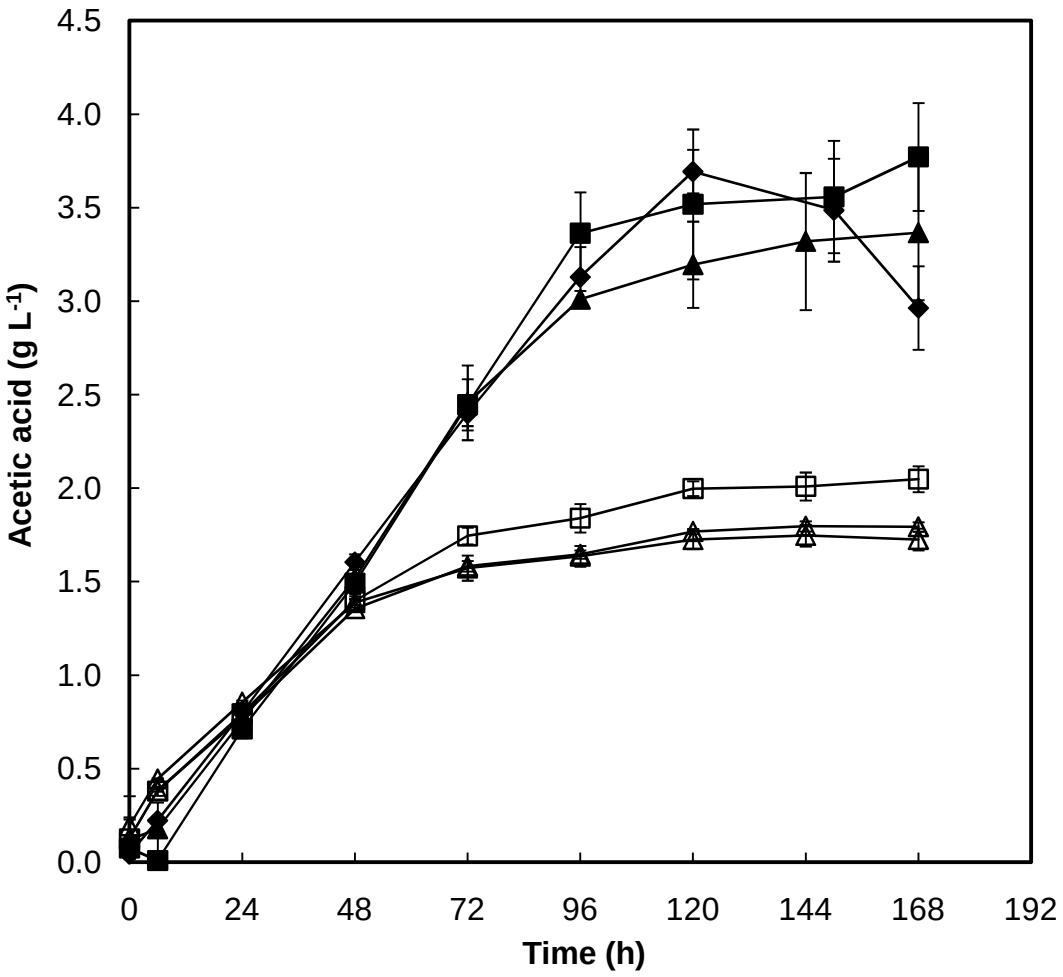

Fig A.9 Acetic acid profiles using *K. marxianus* IMB3 and 8% pretreated switchgrass in SSFs at 45°C and different enzyme loadings (mL g^{-1} glucan): (♦) 0.1, (■) 0.3, (▲) 0.5 ,(◊) 0.7, (□) 0.9, (Δ) 1.1 (n=3).

APPENDIX B

B.1. Measurement of cellulase activity

Enzyme dilutions that were tested: 0.01, 0.00875, 0.0075, 0.005 and 0.00375

$$Activity\ of\ enzyme\ \left(\frac{FPU}{mL}\right) = \frac{0.37}{enzyme\ dilution\ that\ released\ 2mg\ glucose}$$

From logarithmic plot the dilution that released 2 mg of glucose was 0.0045

$$Enzyme\ activity = \frac{0.37}{0.0045} = 82.2\frac{FPU}{mL}$$

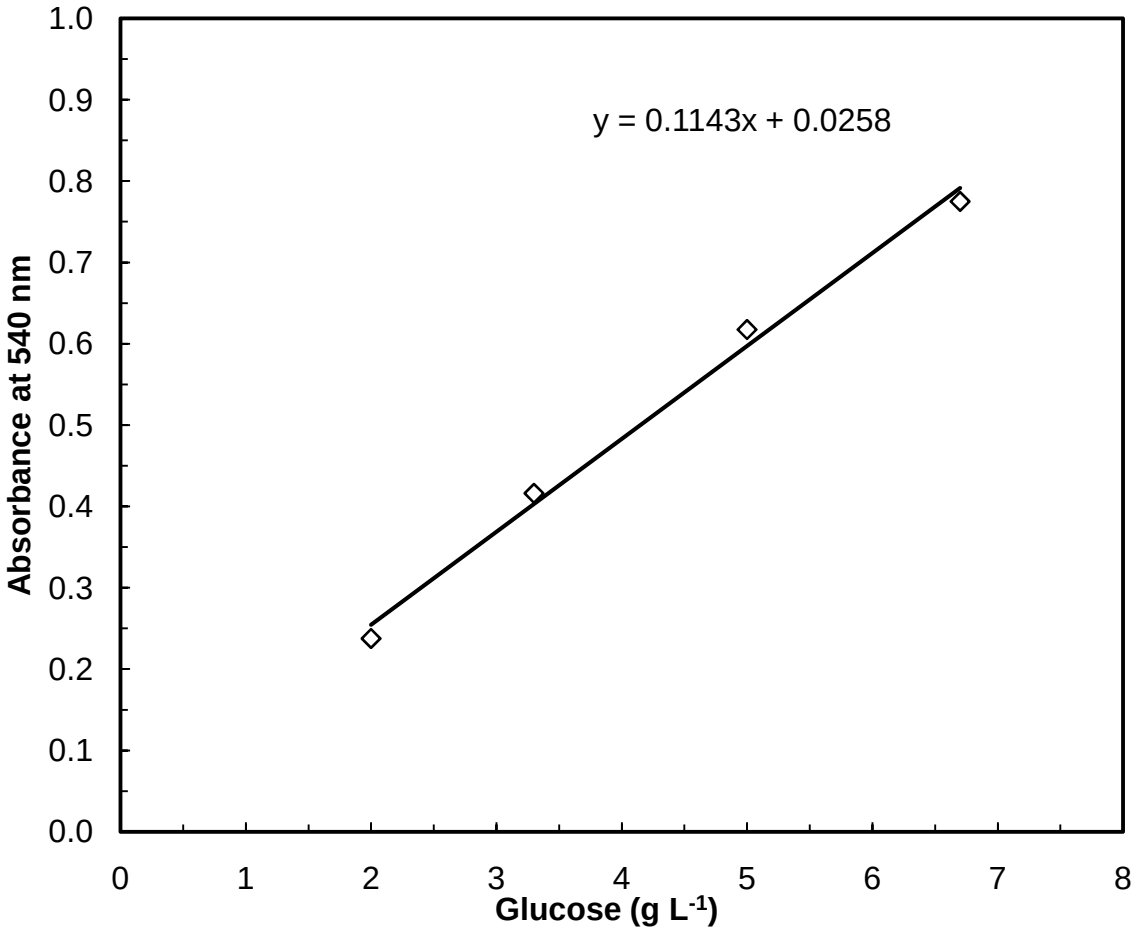

Fig. B.1 Glucose standard curve.

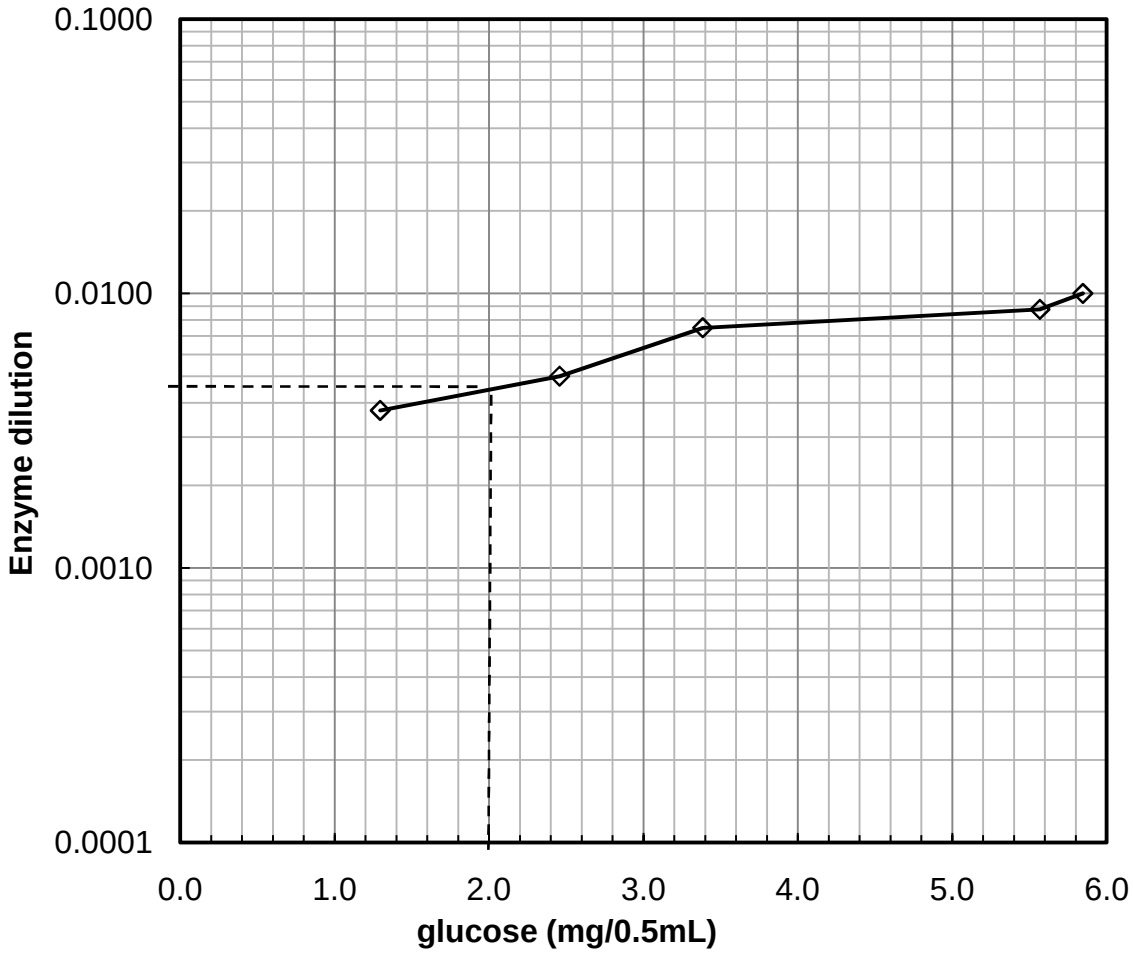

Fig. B.2 Logarithmic plot of glucose concentrations obtained with different enzyme dilutions.

B.2 Sample calculations involved in pretreatment of switchgrass

Moisture content (MC) of switchgrass: 5.6%

Switchgrass to be added in to the reactor: 60 g on dry basis or $60g + (60g * MC/100)$

Switchgrass to be added in to the reactor:

$$60g + \left(60g * \frac{5.6}{100}\right) = 63.36\ g$$

Actual switchgrass loaded: 63.26 g

$$dry\ mass\ of\ grass\ loaded\ (DSL) = \left(1 - \frac{MC}{100}\right) * 63.36g$$

$$dry\ mass\ of\ grass\ loaded\ (DSL) = \left(1 - \frac{5.6}{100}\right) * 63.36g = 60.02g$$

DI water to be added: 540 g; DI water added: 540 g

Wt of prehydrolyzate: 442.29 g

$Weight\ of\ pretreated\ solids$: 133.2 g

Wt of biomass after washing = 122.4 g

% solids recovered = % RS

$$\%RS = \frac{pretreated\ grass\ obtained + mass\ of\ prehydrolyzate}{switchgrass\ added + DI\ water\ added}$$

$$\%RS = \frac{(133.2g + 442.29g) * 100}{(63.36g + 540g)} = 95.4\%$$

$$\%\ solids\ in\ washed\ solids\ (\%SWS) = \left[1 - \frac{A - B}{A - C}\right] * 100$$

Where:

A is the mass of pretreated solids and aluminum pan = 5.456 g

B is the mass of oven dry pretreated grass and aluminum pan = 2.665 g

C is the mass of aluminum pan = 1.500 g

$$\%SWS = \left[1 - \frac{5.456 - 2.665}{5.456 - 1.500}\right] * 100 = 29.5\%$$

$$Pretreated\ solids\ obtained\ on\ dry\ basis(DS) = \ mass\ of\ washed\ solids\ * \ \%SWS$$

$$= 133.2g * 29.5/100 = 39.29g$$

$$\%\ dissolved\ solids = \left[1 - \left(\frac{DS}{DSL} * \%RS\right)\right] * 100$$

$$= \left[1 - \frac{39.29g}{60.02*0.954}\right] * 100 = 31.38\%$$

B.3 Sample calculations involved in acid hydrolysis test

Determination of Owen dry weight (ODW):

$$ODW = \frac{mass\ of\ air\ dry\ sample\ x\%\ total\ solids}{100}$$

$$\%\ total\ solids\text{:}\ \left[1 - \frac{A - B}{A - C}\right] * 100$$

Where: Mass of air dry sample is the mass of air dry solids added in to the pressure tubes = 0.3006 g

A is the mass of sample of air dry pretreated solids and aluminum pan = 2.593 g

B is the mass of oven dry pretreated grass and aluminum pan = 1.0867 g

C is the mass of aluminum pan = 1.5069 g

$$\%\ total\ solids = \left[1 - \frac{2.593 - 1.0867}{2.593 - 1.5069}\right] * 100 = 99.02\%$$

$$ODW = \ 0.3006\left(\frac{99.02}{100}\right) = 0.2977g$$

Mass of crucible: 24.2565 g

Mass of crucible and Acid insoluble residue (AIR): 24.3626 g

Mass of crucible and Ash: 24.2627 g

$$\%AIR = \left(\frac{mass\ of\ crucibles\ plus\ AIR - mass\ of\ crucible}{ODW}\right) * 100 \quad = \left(\frac{24.3626g - 24.2565g}{0.2977}\right) * 100$$

$$\%AIR\ = 35.64\%$$

%Acid insoluble Lignin = % AIL

$$\%AIL =$$

$$[((mass\ of\ crucibles\ plus\ AIR - mass\ of\ crucible) -$$

$$(mass\ of\ crucibles\ plus\ ash - mass\ of\ crucible))/ODW] * 100$$

$$= \left[\frac{[(24.3626g - 24.2565g) - (24.2627g - 24.2565g)]}{0.2977g}\right] * 100$$

$$\%AIL\ = 33.56\%$$

% Acid soluble lignin (%ASL):

$$\%\ ASL = \frac{UVabs * volume\ of\ filtrate * dilution}{\varepsilon * ODW} * 100$$

Where:

UV_{abs} is the average UV-Vis absorbance for sample at 205 nm

Volume of filtrate is 87 mL

$$Dilution = \frac{volume\ of\ sample + volume\ of\ diluting\ solvent}{volume\ of\ sample}$$

$$= \frac{(150ml + 550ml)}{(150ml)} = 4.67$$

ε is the absorptivity of biomass at specific wavelength = 110

$$\% \, ASL = \frac{0.948 * 0.087 * 4.67}{110 * 0.2977} * 100 = 1.17\%$$

$$\% \, lignin \, on \, extractives \, free \, basis = \%AIL + \%ASL = 33.56\% + 1.17\% = 34.73\%$$

Calculations Involved with HPLC determined sugars:

$$\% \, CVS \, recovery = \frac{conc.detected \, by \, HPLC}{known \, conc.of \, standard} * 100$$

Taking glucose in to consideration, conc. of glucose in sugar recovery standards (SRS) = 4.002 g L^{-1}

Conc. of glucose determined by HPLC = 3.447 g L^{-1}

$$\% \, CVS \, recovery = \left(\frac{3.447}{4.001}\right) * 100 = 83.48\%$$

HPLC determined conc. of glucose from acid hydrolysis sample = 1.7901 g L^{-1}

$$Corrected \, concentration \, of \, sugars = \; HPLC \, determined \, conc * \%CVS \, recovery$$

$$= \frac{1.7901}{\left(\frac{83.48}{100}\right)} \; = \frac{2.0919g}{L}$$

Concentration of polymeric sugars prior to hydrolysis

$$C \, anhydro = (Cx) * \; anhydro \, correction + cellobiose \, conc$$

Anhydro correction is 0.9 for C5 sugars and 0.88 for C6 sugars.

$$C \, anhydro = \left(2.0919 * \frac{162}{180}\right) + 0.0084 \; = \frac{1.9667g}{L} \; glucan$$

$$\%S\ ext\ free\ = (C\ anhydro * \ Volume\ of\ filtrate) * \frac{100}{ODW}$$

%S ext free is the % sugars on extractives free basis.

For glucan:

$$\%S\ ext\ free = \frac{[1.9667*0.087*100]}{0.2977} = 57.7\%$$

B.4 Sample calculations involved in SSF

% solids in pretreated switchgrass = 29.5, which is determined as mentioned in sample calculations in pretreatments section.

Total mass inside the flask=100 g

Desired solid loading (%w/v) = 8

Glucan dry wt = 57.7% as obtained from acid hydrolysis test.

Glucan present in 8% solids (%g g^{-1}) = 4.62%

$$Switchgrass\ needed = \% \frac{solids}{\%total\ solids\ in\ switchgrass} = \frac{8}{\frac{29.5}{100}}$$

$$Switchgrass\ needed = 27.12g$$

Switchgrass added to the flask: 27.12 g

Desired cellulase loading: 0.7 mL g^{-1}; Activity of enzyme=82.2 FPU mL^{-1}

$$Cellulase\ added = \ Actual\ glucan\ loaded * enzyme\ loading\ per\ gram\ glucan$$

$$= 4.62 * 0.7 = 3.23\ ml$$

1M citrate buffer added = 5 mL; 10X media=10 mL; 100X inoculum (OD of 56) = 1 mL

$Water\ to\ be\ added \quad =$

$100 - (actual\ switchgrass\ loaded - cellulase\ added - citrate\ buffer\ added - media\ added - inoculum\ added)$.

$Water\ added = 100 - (27.12g - 3.23mL - 5mL - 10mL - 1mL) = 55.4g$

Mass of flask before autoclaving and after autoclaving is noted as 242.53 and 238.96 g, respectively

$Corr.Sterile\ DI\ water\ added = 242.53 - 238.96 = 3.57mL$

Theoretical yield of ethanol:

$$= \left(0.51 * \left(\% \frac{Glucan}{100}\right) * (Actual\ sample\ loaded\ ) * \left[\frac{\frac{\%total\ solids}{100}}{\frac{total\ mass}{1000}}\right] * 1.111\right)$$

$$= \left(0.51 * \left(\frac{57.7}{100}\right) * \left(27.12g * \left[\frac{\frac{29.5}{100}}{\frac{100g}{1000}}\right] * 1.11\right)\right)$$

$=26.156$ g L^{-1}

$\%\ cellulose\ conversion: \left(\frac{[corrected\ ethanol\ obtained]}{[theoretical\ yield\ of\ ethanol]}\right) * 100$

Corrected ethanol is ethanol obtained in flask minus ethanol obtained in control at that time point (here 0.7mL g^{-1} enzyme loading at 45°C, 24 h)

$\%\ cellulose\ conversion = \left(\frac{18.139 - 0.071}{26.156}\right) * 100 = 69.35\%$

Calculating the volume of culture required for inoculation:

OD of culture =0.8324 with dilution factor as 5.88

$$OD\ of\ flask = \left(\frac{100}{df}\right) * OD = 100 * \frac{0.8324}{5.88} = 14.15$$

$$OD\ of\ flask\ = 100 * \frac{0.8324}{5.88} = 14.15$$

Volume of culture required to obtain 100X cells:

$$Vol = SSF\ vol * \frac{(initial\ OD\ in\ SSF\ flasks)(no.of\ SSF\ flasks+1)}{OD\ of\ flask}$$

$$Vol\ required = 100mL * 0.56 * \frac{13}{14.15} = 51.5mL$$

B.5 Sample calculations for mass balance on pretreatment

Switchgrass before pretreatment was composed of 41.9% glucan, 25.1% xylan, 0.7% galactan, 2.2% arabinan.

Glucose, xylose, lignin present in 60 g of dry switchgrass that was added to the PARR reactor:

$$Glucose\ present\ = switchgrass\ added\ to\ PARR\ reactor * \left(\%\frac{glucan}{100}\right) * 1.11$$

$$Glucose\ present\ = 60 * \left(\frac{41.9}{100}\right) * 1.11 = 27.91\ g$$

$$xylose\ present\ = switchgrass\ added\ to\ PARR\ reactor * \left(\%\frac{xylan}{100}\right) * 1.11$$

$$xyloe\ present\ = 60 * \left(\frac{25.1}{100}\right) * 1.11 = 16.7\ g$$

$$lignin\ present\ = switchgrass\ added\ to\ PARR\ reactor * \left(\%\frac{lignin}{100}\right) * 1.11$$

$$Lignin\ present\ =\ 60 * \left(\frac{21}{100}\right) * 1.11 = 12.6\ g$$

It was assumed that 125 g (wet basis) of switchgrass was obtained after pretreatment and contained 70.5% moisture or 29.5% solids. About 450 g of prehydrolyzate is obtained after pretreatment and contained 3.4 g L^{-1} glucose, 15.2 g L^{-1} xylose, 0.45 g L^{-1} xylitol, 3.4 g L^{-1} acetate, 0.037 g L^{-1} glycerol and other products.

Amount of Glucose, xylose and lignin present in pretreated solids:

$$Glucose\ present =\ switchgrass\ after\ pretreatment * \left(\%\frac{solids}{100}\right) * \left(\%\frac{glucan}{100}\right) * 1.11$$

$$Glucose\ present\ =\ 125 * 0.295 * \left(\frac{58}{100}\right) * 1.11 = 23.74\ g$$

$$Xylose\ present =\ switchgrass\ \ after\ pretreatment * \left(\%\frac{solids}{100}\right) * \left(\%\frac{Xylan}{100}\right) * 1.12$$

$$xylose\ present\ =\ 125 * 0.295 * \left(\frac{5}{100}\right) * 1.12 = 2.06\ g$$

$$Lignin\ present =\ switchgrass\ after\ pretreatment * \left(\%\frac{solids}{100}\right) * \left(\%\frac{lignin}{100}\right)$$

$$lignin\ present\ =\ 125 * 0.295 * (5/100) * 1.11 = 12.91\ g$$

Glucose used for formation of acetate, glycerol:

$$Glucose\ used\ =\ \left(\frac{acetate}{1}\right) + \left(\frac{glycerol}{0.51}\right)$$

$$Glucose\ used\ =\ \left(\frac{3.4}{1}\right) + \left(\frac{0.037}{0.51}\right) =\ 3.42\ g$$

$$Xylose\ used\ for\ formation\ of\ xylitol\ = \frac{xylitol}{0.51}$$

$$Xylose\ used\ for\ formation\ of\ xylitol\ = \frac{0.45}{0.51} = 0.23$$

$$Total\ glucose\ monomers\ obtained\ =$$

$$glucose\ present\ in\ solids + \ glucose\ in\ prehydrolyzate$$

$$Total\ glucose\ monomers\ obtained\ =\ 23.74 + \ 3.4\ =\ 27.14\ g$$

$$Total\ xylose\ monomers\ obtained\ =\ xylose\ present + \ xylose\ in\ prehydrolyzate$$

$$Total\ xylose\ monomers\ obtained\ =\ 2.06 + \ 15.2\ =\ 17.26\ g$$

$$Glucose\ balance\ = \frac{glucose\ monomers\ obtained + glucose\ used\ for\ product\ formation}{glucose\ present\ in\ switchgrass\ before\ pretreatment}$$

$$Glucose\ balance\ =\ (27.14 + 3.42) * \frac{100}{27.91} =\ 109.5\%$$

$$xylose\ balance\ = \frac{xylose\ monomers\ obtained + xylose\ used\ for\ product\ formation}{xylose\ present\ in\ switchgrass\ before\ pretreatment}$$

$$xylose\ balance\ =\ (17.27 + 0.23) * \frac{100}{16.87} =\ 103.7\%$$

Water balance in pretreatment process:

Water added to pretreatment reactor = 540 g

$$Water\ in\ solids\ =\ solids\ obtained\ after\ pretreatment * moisture\ \frac{content}{100}$$

$$Water\ in\ solids\ =\ 125 * \frac{70.5}{100} = 88.1g$$

$$Water\ loss = \ (prehydrolyzate\ +\ water\ in\ solids) * 100/(water\ added)$$

$$Water\ loss = \ (450\ g\ +\ 88.1g) * \frac{100}{540g} = 0.35\ \%$$

B.6 Sample calculations for mass balance on SSF

This is done for the effect of enzyme loading experiment with enzyme loading 0.7 mL g^{-1} glucan at 45°C.

Products obtained with 8% solid loading and 0.7 mL g^{-1} glucan at 45°C were (g L^{-1}):

ethanol 22.2 , acetic acid 3.57, glycerol 3.4, succinic acid 0.71, xylitol 1.22, xylose1.3, cellobiose 0.24 and glucose, 2.059.

Availability of glucose, xylose and lignin initially:

$$Available\ glucose\ = 80 * 0.58 * 1.11 = 51.5 \frac{g}{L}$$

$$Available\ xylose\ = 80 * 0.05 * 1.12\ = 4.48 \frac{g}{L}$$

$$Available\ lignin\ = 80 * 0.35\ = 28 \frac{g}{L}$$

% glucose consumed for products (ethanol, acetic acid, glycerol and succinic acid):

$$\%\ glucose\ consumed = \left(\frac{\left[\left(\frac{EtOh}{0.51} \right) + \left(\frac{acetic\ acid}{1} \right) + \left(\frac{glycerol}{0.51} \right) + \left(\frac{succinic\ acid}{1.31} \right) \right]}{51.5} \right) * 100$$

$$\%\ glucose\ consumed = \left(\frac{\left[\left(\frac{22.1}{0.51} \right) + \left(\frac{3.57}{1} \right) + \left(\frac{3.4}{0.51} \right) + \left(\frac{0.71}{1.31} \right) \right]}{51.5} \right) * 100$$

$$\%\ glucose\ consumed = \ 105.4\%$$

$$\%\ glucose\ accounted = \left[\left(residual\ glucose + (residual\ cellobiose * 1.05) \right) * \frac{100}{46.4} \right] + \%\ glucose\ consumed$$

$$\%\ glucose\ accounted = \left[\left(2.059 + (0.24 * 1.05)\right) * \frac{100}{46.4}\right] + \%\ glucose\ consumed$$

% glucose accounted = 110.42%, which was the glucose balance that was used in the discussion.

$$\%\ xylose\ accounted\ = \left(xylose + \left(\frac{xylitol}{0.51}\right)\right) * \frac{100}{4.48}$$

Where 1.3 was the concentration of xylose measured after 168 h in SSF.

$$\%\ Xylose\ accounted\ = \left(1.3 + \left(\frac{1.22}{0.51}\right)\right) * \frac{100}{4.48} = 82.4\%$$

% xylose accounted = 82.4%, which was the xylose balance that was used in the discussion.

Glucan conversion efficiency calculation:

It was assumed that 20% of solids were removed due to sampling and 60% of solids were dissolved during SSF.

Initial glucan, xylan and lignin composition of solids at the beginning of SSF:

glucan: 58%, Lignin :35%, Xylose: 5%

$$Glucan\ present\ in\ the\ begining\ of\ SSF\ =\ conc\ of\ solids * \%\ glucan\ in\ solids$$

$$Glucan\ present\ in\ the\ beginning\ of\ SSF\ =\ 80 * \frac{58}{100} =\ 46.4\ \text{g}$$

$$xylan\ present\ in\ the\ begining\ of\ SSF\ =\ conc\ of\ solids * \%\ glucan\ in\ solids$$

$$xylan\ present\ in\ the\ beginning\ of\ SSF\ =\ 80 * \frac{5}{100} =\ 4g$$

**The values were rounded off to the nearest whole number.

Final glucan and lignin composition of solids at the end of SSF: % glucan, xylan and lignin after acid hydrolysis test of SSF samples were 4.67 %, 0.0 % and 72.7 %.

Glucan left in the flasks after SSF:

$$Glucan\ left\ =$$

$$(conc\ of\ solids\ in\ SSF\ at\ the\ begining) *$$

$$(100\ -\ \%\ solids\ removed\ from\ SSF\ due\ to\ sampling) *$$

$$(\%\ solids\ dissolved\ \ during\ SSF) * \frac{\%\ glucan\ in\ switchgrass\ after\ SSF}{100}$$

$$Glucan\ left\ =\ 80 * 0.8 * 0.4 * 4.67/100\ =\ 1.19\ g/L$$

$$Lignin\ left\ =\ (\ conc\ of\ solids\ present\ in\ SSF\ at\ the\ begining) * (100\ -$$

$$\%\ solids\ removed\ from\ SSF\ due\ to\ sampling) *$$

$$(\%\ solids\ dissolved\ \ during\ SSF) * \frac{\%\ lignin\ in\ switchgrass\ after\ SSF}{100}$$

$$Lignin\ left\ =\ 80 * 0.8 * 0.4 * 72.7/100\ =\ 27.9\ g/L$$

$$Glucan\ conversion\ efficiency\ =\ (initial\ glucan\ present - glucan\ left) *$$

$$\frac{100}{initial\ glucan\ present}$$

$$Glucan\ conversion\ efficiency\ =\ (46.4 - 1.19) * \frac{100}{46.4} =\ 97.4\%$$

$$Lignin\ balance\ = (initial\ lignin) * \frac{100}{lignin\ left}$$

$$Lignin\ balance\ =\ 27.9 * \frac{100}{28} = 99.67\%$$

VITA

Naveen Kumar Reddy Pessani

Candidate for the Degree of

Master of Science

Thesis: SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF SWITCHGRASS BY THERMOTOLERANT *KLUYVEROMYCES MARXIANUS* IMB3: EFFECT OF ENZYME LOADING, TEMPERATURE AND OPERATING MODE

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Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF SWITCHGRASS BY THERMOTOLERANT *KLUYVEROMYCES MARXIANUS* IMB3: EFFECT OF ENZYME LOADING, TEMPERATURE AND OPERATING MODE

Pages in Study: 102 Candidate for the Degree of Master of Science

Major Field: Biosystems Engineering

Scope and Method of Study:

The objectives of this research were to determine the optimum enzyme (Accellerase 1500, Genencor International, NY, USA) loading, temperature and operating modes (batch and fed-batch) on ethanol production by simultaneous saccharification and fermentation (SSF) of pretreated Kanlow switchgrass using thermotolerant *Kluyveromyces marxianus* IMB3. Hydrothermolysis pretreatment of switchgrass was used. Also, 8% solids (dry basis, db) loading was used for the effect of enzyme loadings and temperatures tests. Various loadings of Accellerase 1500 (0.3, 0.5 and 0.7 mL g^{-1} glucan) were used in SSF at 45°C (activity of enzyme was 82.2 FPU mL^{-1}). Then, SSFs were performed at 37, 41 and 45°C with the optimum enzyme loading. Finally, four different batch and fed-batch strategies were evaluated at the optimum enzyme loading and temperature with a total solid loading of 12% (db).

Findings and Conclusions:

The optimum enzyme loading was 0.7 mL g^{-1} glucan in SSF with 8% solids at 45°C, which resulted in the highest ethanol concentration of 22.3 g L^{-1}. This was equivalent to 85% maximum theoretical yield (MTY). For the effect of temperature experiment, the highest ethanol concentration of 23.0 g L^{-1} (86.3% MTY) was obtained in SSF with 8% solids at 45°C compared to about 20 g L^{-1} ethanol (77% MTY) obtained at both 37°C and 41°C. In addition, the results showed that about 32 g L^{-1} ethanol (81% MTY) was produced with a total solid loading of 12% (db) in all batch and fed-batch SSFs strategies. No significant differences in the amount of ethanol produced in batch or fed-batch operating modes were observed in the range of solids and enzyme loadings used ($p > 0.05$). Moreover, the results also showed that with fed-batch mode and 12% solids (db), the enzyme loading can be decreased by 33% of the optimum loading for batch process.

ADVISER'S APPROVAL: Dr. Hasan K. Atiyeh